Biology and therapeutic potential of enteric nervous system stem cells for spinal cord injury

By

Benjamin S. Jevans

Report submitted to University College London for the degree of doctor of philosophy

2017

Stem Cells and Regenerative Medicine

UCL Great Ormond Street Institute of Child Health

Declaration:

I, Benjamin Stuart Jevans, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Spinal cord injury (SCI) is one of the leading and most crippling causes of acquired neurological impairment. It affects around 40,000 individuals in the UK, causing paralysis, multisystem impairment and a reduced life expectancy. This has a devastating impact on quality of life and places stress upon both individuals and healthcare systems. Despite extensive characterization of SCI pathology, there remains no cure. Stem cells offer a potential therapy since they can replace lost neurons, promote axonal regeneration and limit scar formation, but an optimal stem cell source has yet to be found. Enteric neural stem cells (ENSCs) are a possible solution. ENSCs comprise the renewing population of the enteric nervous system (ENS), the intrinsic innervation of the gastrointestinal (GI) tract. ENSCs can be isolated from the GI tract via minimally invasive techniques such as endoscopy, and when transplanted into aneural or dysfunctional gut, are capable of reforming a functional ENS. This thesis assesses the potential of ENSCs as a stem cell source for SCI.

Chimeric neural tube grafting was used to fluorescently label chick ENSCs, allowing for isolation and lineage tracing. *In vitro* characterization revealed neuronal subtypes that were common between ENSCs and spinal cord tissue. Following transplantation into the embryonic chick spinal cord ENSCs survived, differentiated into neurons and formed anatomical bridges across the injury zone. In an adult rat model of SCI, ENSC transplantation was combined with application of chondroitinase ABC (ChABC), a modifier of the inhibitory microenvironment observed within SCI. ENSCs, when transplanted on their own, demonstrated extended survival, differentiated into neurons and bridged the injury site. Combined treatment (ENSCs + ChABC) resulted in a significant improvement in lesion histology, including reduction of the injury cavity, and increased numbers of endogenous axons crossing the injury

site. Together, these findings establish ENSCs as a promising alternative stem cell source for SCI.

Acknowledgements

I knew that this was the right position for me from the moment I read the project abstract, so my first thanks have to go to Doctors Alan Burns and Nikhil Thapar for trusting me with their idea. Placing this project into my hands and giving me so much free reign to explore it has made me the researcher I am today (not that I'm trying to assign blame), and for that I will always be truly grateful. Second thanks go to the Anatomical Society for generously, funding this work.

I have been supported immensely in this project by Doctors Conor McCann, Dipa Natarajan and Julie Cooper. This experience was made all the richer for you being a part of it. The curious combination of making me feel I could achieve this, pointing out when I was a 'numpty', and providing endless advice and proof reading, somehow helped me limp along to the finish line. I wouldn't have chosen anyone else and I'll miss you all.

To my little sister, who is so delighted to know that I will now be "Dr BJ". You have helped more than you know by always being on the other end of the phone and showing me the importance of family. To my brother, for setting the bar and pushing me, without realizing it, to achieve what I have. Thank you both. I also could never have embarked on this career path without the support of my parents. You encouraged me to follow my dreams, so really I guess this is your fault for not directing me to a career with more job security, a reasonable income and stable career progression. Thank you.

To Hannah. You have calmed me down countless times, guided me to an answer and made me feel ok about it (it worked out fine almost every time, I'll let you off for the others). Having you to lean on made all the difference.

There are so many other family members, friends and colleagues who helped me get through the last three years. I can't name you all, but I endlessly appreciate you supporting and tolerating me.

And to my Grandad, Reginald Thomas George Jevans, the kindest man I have ever known. This is, and always has been, for you.

Table of contents:

List of figures

List of Tables

Abbreviations

5-HT	Serotonin
A/P	Anterior posterior
ACh	Acetylcholine
AFSC	Amniotic fluid stem cells
BDA	Biotinylated dextran amine
BrdU	Bromodeoxyuridine
BSJ	Benjamin Stuart Jevans
C	Cervical
CCD	Charge-coupled device
cDNA	Complementary deoxyribonucleic acid
ChABC	Chondroitinase ABC
ChAT	Choline acetyltransferase
CNS	Central nervous system
CSPG	Chondroitin sulphate proteoglycans
D/V	Dorsal ventral
DAB	Diaminobenzidine
DAPI	Diamidinophenylindole
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DPI	Days post injury
DPX	Distyrene tricresyl phosphate xylene
DRG	Dorsal root ganglia
E	Embryonic day
EB	Emily Burnside
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
eGFP	Enhanced GFP
ENCC	Enteric neural crest cell
ENS	Enteric nervous system
ENSC	Enteric neural stem cell
EtOH	Ethanol
FACS	Fluoresence activated cell sorting
FBS	Fetal bovine serum
FG	Fluorogold
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	Forelimb
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GI	Gastrointestinal
GLS1	Glutaminase 1
H&E	Haemotoxylin and eosin
HCl	Hydrochloric acid
HL	Hindlimb
HNK1	Human natural-killer 1
HRP	Horseradish peroxidase
Iba1	Ionized calcium-binding adaptor molecule 1
iPSC	Induced pluripotent stem cells
iPSCMN	Induced pluripotent stem cell motoneurons
L/R	Left right
MYP	Myenteric plexus

NC	Neural crest
NCC	Neural crest cell
NJ	Nick James
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOR	Nerve of Remak
NSC	Neural stem cell
NSM	Neural stem cell media
NSM+P	Neural stem cell media + growth factors
NT	Neural tube
OCT	Optimal cutting temperature
OEC	Olfactory ensheathing cells
OEG	Olfactory ensheathing glia
P	Postnatal day
$_{P}$75	Low-affinity nerve growth factor receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PNS	Peripheral nervous system
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RT	Room temperature
SC	Spinal cord
SCI	Spinal cord injury
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIN	Self-inactivating
SMP	Submucosal plexus

SN	Spinal nerve
SOX	Sry-related box
T	Thoracic
TAE	Tris base, acetic acid, and EDTA
TPH	Tryptophan hydroxylase
TuJ1	Class III β-Tubulin
WT	Wild type

General Introduction

1.1 Spinal cord injury (SCI) background

SCI has an estimated UK prevalence of around 40,000 (Gall and Turner-Stokes, 2008). Twenty to forty year old males are the most commonly affected, with motor vehicle collisions or sporting accidents as the leading cause of injury (Nas et al., 2015). Sufferers typically experience paralysis from the point of injury down (cervical injuries comprise the majority), multisystem impairment, and reduced life expectancy (Sezer et al., 2015). The life expectancy of suffers is rising alongside better care standards, but this is revealing additional problems as SCI patients begin to experience cardiovascular issues and other age-related illnesses, the treatment of which is complicated by (most commonly) tetraplegia (Gall and Turner-Stokes, 2008; Hachem et al., 2017). In addition to the numerous physical symptoms, associated psychosocial factors resulting from the injury severely impact on sufferer's mental well-being (Cao et al., 2014). SCI patients have increased rates of depression and anxiety (Craig et al., 2017; Lim et al., 2017). This has translated to increased rates of suicide among SCI patients, the incidence of which has risen while other causes of death have fallen (Soden et al., 2000). Among factors such as reduced self-esteem and frustration, chronic pain is thought to impact on these rates (Ruan and Luo, 2017). Taking into account both the physical and mental effects of SCI, patients have a significantly lower quality of life than the healthy population (Moreno et al., 2017; Palimaru et al., 2017; Sweet et al., 2014). SCI also poses a significant financial burden to the patient (in terms of lost earning potential, home adaptations and living assistance) and on the National Healthcare System.

SCI has an immense impact on the physical, mental and social health of both patients and the community, an effect that could be lessened by even partial restoration of one or more functions lost through SCI (Anderson, 2004;

Simpson et al., 2012). However, despite extensive research, no treatment options are currently available to restore complete motor and sensory functions following SCI (Rouanet et al., 2017). Therefore, the development of more effective treatments, or better yet, cures for SCI is of paramount importance.

1.2 Spinal cord development

The spinal cord serves as the interface between the brain and the rest of the body. It is the hub where the central and peripheral nervous systems (CNS and PNS, respectively) meet to allow seamless communication. Indeed, while the CNS and PNS are often thought of as isolated entities, the two in reality form a continuous structure, with all nervous tissues originally derived from a single germ layer, the ectoderm (Wittler and Kessel, 2004).

The CNS is established first during early embryonic development via the process of neurulation (Fig. 1.1) (Gammill and Bronner-Fraser, 2003). This process begins during the trilaminar stages of embryogenesis, initiated by the notochord inducing the overlying ectoderm to form the neurectoderm (Yamada et al., 1991). A small furrow, the neural groove, forms in the neurectoderm along the anterior-posterior axis (Colas and Schoenwolf, 2001). The two edges of the neurectoderm then rise to form the neural folds, which meet and fuse to form the neural tube (Pai et al., 2012). Initially consisting of a single cell layer, the germinal neuroepithelium (Sauer, 1935), undergoes progressive cell division (mostly in the dorsal aspect of the tube) to give rise to a laminated structure consisting of the germinal, mantle and marginal zones. While changing greatly in appearance, this early structure is maintained throughout life, with the germinal, mantle and marginal zones developing into the ependymal canal, and grey and white matter of the adult spinal cord, respectively (Fig. 1.2) (Gilbert, 2000).

Throughout development and adulthood these structures remain anatomically and functionally distinct. The ependymal canal, formed of the early germinal neuroepithelium, retains a modest stem cell pool late into adulthood (Panayiotou and Malas, 2013). The grey matter, centralised within the cord (in contrast to the brain), contains the cell bodies of spinal neurons. The white matter is mostly occupied by axons projecting from these neurons or entering from sensory neurons of the PNS (Gilbert, 2000). These layers stretch the entire length of the neural tube, the progressive folding of which forms all aspects of the CNS.

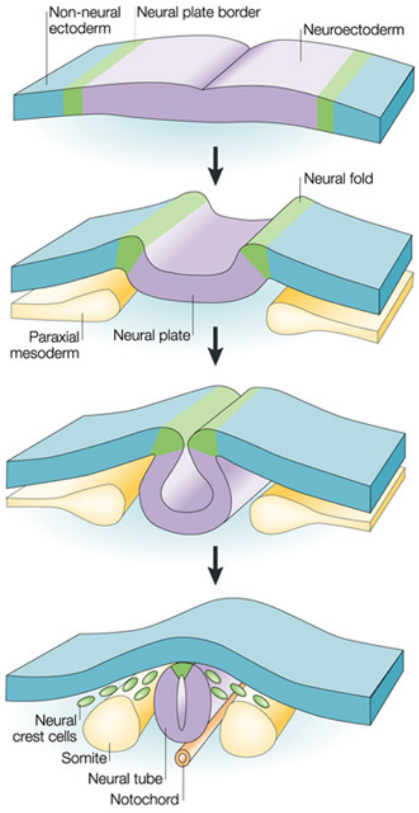

Figure 1.1: The spinal cord and neural crest are formed through the process of neurulation early in development.

Neurulation begins with induction of the neural plate by the underlying notochord. This folds along the anterior-posterior axis to form the neural groove, the outer edges of which rise to form the neural folds. These gradually meet and fuse to initially give rise to a single cell layered structure, the neural tube. Neural crest cells are formed at the boundary between the neural tube and the non-neural ectoderm. Taken from (Gammill and Bronner-Fraser, 2003).

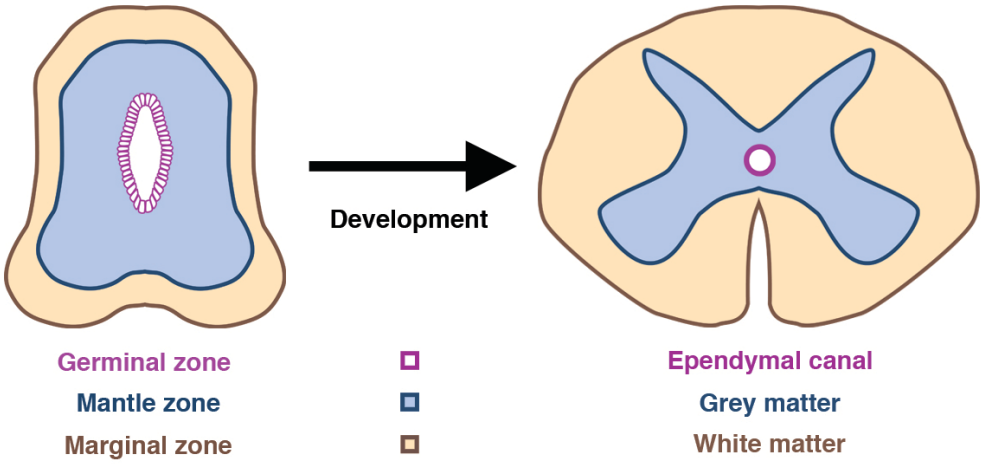

Figure 1.2: The trilaminar structure of the developing neural tube is mirrored in the adult spinal cord.

The neural tube is formed of neuoectoderm. In the early stages of neural tube formation, early progenitor cells within the CNS - the neuroepithelial cells of the germinal zone (pink) – give rise to the mantle (blue) and marginal zone (brown). The mantle zone consists of neuroblasts that will form the future grey matter of the spinal cord. These cells send out projections into the marginal zone, which forms the white matter of the adult spinal cord. This contains the vast majority of ascending sensory and descending motor tracts of the CNS.

Ependymal cells of the early germinal neuroepithelium give rise to almost all neuronal and glial cell types throughout the mature spinal cord (Kriegstein and Alvarez-Buylla, 2009). Neuroepithelial cells give rise to the radial glia, themselves multipotent progenitors (Huttner and Kosodo, 2005; Noctor et al., 2004). Through a series of symmetric and asymmetric divisions, radial glia give rise to the neurons and glia of the future spinal cord (Paridaen and Huttner, 2014).

In the developed spinal cord, these progenitor–derived cells assume specific functions. Spinal neurons are responsible for the transmission of

sensory and motor information between the brain and body. All such cells are multipolar, including efferent, projection and interneurons (Caspary and Anderson, 2003; Gouti et al., 2015). They are capable of producing/ responding to a range of neurotransmitters as well as noxious stimuli, allowing them to facilitate reflex arcs and limit damage sustained from the external environment. However, neurons cannot carry out these functions in isolation. Glia, the most common cell type in the CNS and originally thought to be nothing more than a glue to hold neurons in place, are now known to be as equally important as neurons for correct functioning of the CNS (Gonzalez-Perez et al., 2015). As well as giving rise to the first neurons, radial glia are vital for axon guidance and proper spatial development of the grey matter (McDermott et al., 2005). Oligodendrocytes myelinate the neurons to enhance axonal conduction (Bradl and Lassmann, 2010), and astrocytes take part in the tripartite synapse (among numerous other roles) to facilitate efficient synaptic transmission (Perea et al., 2009). Microglia (the exception to CNS genesis) are generated from blood (Ginhoux et al., 2010) and function to ensure immune stability of the spinal cord microenvironment and remove dead cells/ debris.

In health, these neurons and glia function seamlessly to allow the spinal cord to carry out a wide range of functions. As well as enabling reflex arcs, the adult spinal cord is involved in almost every aspect of bodily function. The cord contains a series of ascending sensory and descending motor pathways between the brain and body, to allow interaction with the external environment, and monitoring/ modulation of the internal environment. These pathways are organised into numerous tracts and often extend over considerable distances (Levy et al., 2015). Severing of these tracts, in many cases, is both catastrophic and irreversible.

1.3 Spinal cord injury

While originally derived from a common cell source, the response of the PNS and CNS to injury is remarkably distinct. Unlike the PNS, the regenerative capacity of the CNS is severely limited (Huebner and Strittmatter, 2009). Trauma to the spinal cord therefore leads to an irreversible loss of neurons, inevitably causing destruction of ascending and descending axonal tracts. Compounding matters, the injury response processes that apparently exist to limit the spread of the resulting lesion actually appear to perturb regeneration. The cascade of events occurring following SCI can be grouped into two phases. The first consists of necrotic cell death directly related to the spinal cord trauma (McDonald and Sadowsky, 2002). The secondary phase begins immediately after this insult, and comprises reactive, ongoing processes which hinder endogenous regeneration (Tator, 1991). Secondary processes can continue for months (summarised in Fig. 1.3), and are associated with continuing deterioration of the patient's condition.

The initial mechanical damage to the spinal cord results in both necrotic death of neurons and the axonal interruption of many surviving neurons. This leads to Wallerian degeneration – degradation of the axon distal to the injury - and retraction of the proximal axon (Vargas and Barres, 2007). This axonal insult also leaves the surviving cell body vulnerable to apoptotic death (Ruff et al., 2008). In addition, the injury causes haemorrhage from ruptured blood vessels supplying the cord and disruption of the blood-spinal cord barrier. These events lead to the secondary phase of spinal cord injury.

Many blood components are neurotoxic (Mautes et al., 2000), leading directly to cell death from contact with blood products, and indirectly causing hypoxia/ death of cells previously supplied by the severed vessels.

This also leads to significant inflammation, arguably the most harmful aspect of secondary injury, involving the invasion of the spinal cord by macrophages, neutrophils and T-cells (Zhang et al., 2012). Along with activated resident microglia, these invading cells release free radicals and other noxious substances, compounding the ongoing cell death (David and Kroner, 2011; Nguyen et al., 2007). Astrocytes within the spinal cord are also recruited to the site of injury, where they proliferate around the lesion and, in turn' recruit fibroblasts to form a 'glial scar' (Sun and Jakobs, 2012). While this is likely a protective mechanism to prevent further necrotic spread from the injury site, the scar is now known to also present both a physical and chemical barrier to regenerative cell migration/ axon extension across the lesion. Astrocytes within the scar produce extracellular matrix proteins such as chondroitin-sulfate proteoglycans (CSPGs) (Burnside and Bradbury, 2014; Morgenstern DA et al., 2002), which induce axonal growth cone collapse and prevent attempts by neurons to project axons across the injury site. Unsupported cells within the lesion site continue to die/degenerate. Combined, these processes result in the formation of fluid-filled cavities walled by glial scar tissue (Fig. 1.3).

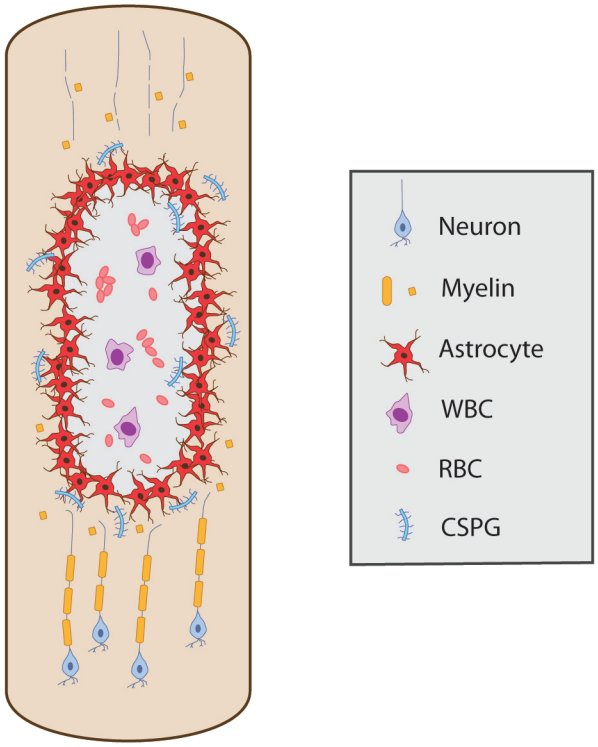

Figure 1.3: Secondary mechanisms of spinal cord injury compound cell death and hinder endogenous recovery.

Following mechanical injury, the severing of blood vessels leads to haemorrhage, inflammation and oedema, as well as ischemia of target tissues of the disconnected vessels. Severed axons undergo demyelination and Wallerian degeneration, releasing inhibitory debris into the injury microenvironment. These processes result in the formation of fluid-filled cavities within the cord, which are quickly enveloped by the glial scar. This contains astrocytes and fibroblasts, and presents a physical and chemical barrier to regeneration (Mothe and Tator, 2012).

By-products of the injury process, such as CSPGs produced by astrocytes, quickly form a chemically inhibitory environment towards regeneration. Despite the extensive invasion of phagocytotic cells, there is poor clearance of various inhibitory factors from the injury site (Fawcett and Asher, 1999). Myelin released from dying oligodendrocytes is one such factor, and its degradation produces several substances detrimental to axon growth (McGee and Strittmatter, 2003). Evidence for the inhibitory properties of myelin have been provided by Liebscher *et al* and Merkler *et al*, who demonstrated significant functional recovery of SCI rodents following treatment with monoclonal antibodies raised against myelin-based products

(Liebscher et al., 2005; Merkler et al., 2001). Furthermore, onset of myelin production during chick embryo development coincides with the stage at which the spinal cord can no longer recover from injury (Macklin and Weill, 1985).

Much of the spinal cord's inability to functionally regenerate after injury is attributed to this inhibitory microenvironment. *In vitro* it has been demonstrated that CNS cells are capable of axonal regeneration if placed in more permissive culture conditions (Taylor et al., 2005), and CNS axons have been shown to extend into peripheral nerve grafts following SCI (Richardson et al., 1980). In addition, when placed alongside neurons isolated from the PNS or in supportive scaffolds, CNS neurons have demonstrated extension of axons and establishment of new connections (Meng et al., 2014), implying that surviving neurons are intrinsically capable of regeneration, but their surroundings render such recovery impossible.

As well as severe difficulties in projecting axons across the injury to restore connections, the spinal cord appears unable to replace cells lost through injury. While stem cells within the central canal have been shown to proliferate in response to SCI, the cells formed are almost entirely glial, and appear to contribute the majority of cells that form the glial scar (Barnabe-Heider et al., 2010). Attempts to stimulate additional proliferation, or to encourage neuronal differentiation, have led to modest improvements in functional recovery (Corns et al., 2015; Kojima and Tator, 2002). However, the mechanisms underlying this regeneration are unclear, especially as these attempts have mostly resulted in further glial production.

These secondary events reveal a host of complicated issues that will need to be addressed by any attempt at a therapy for SCI. In order to preserve surviving endogenous neurons, the inhibitory environment, including inflammation, must be in some way made more permissive. This

will likely support endogenous regeneration, including preservation of injured tissue and extension of axons across the injury site. However, the fluid-filled cavities leave little in the way of substrates for axonal regeneration, an issue which must also be addressed. Additionally, any attempt to stimulate regeneration would be aided by replenishment of the lost cell population. Considering the difficulties of stimulating neuronal production from resident stem cells in the ependymal canal, it is likely that any attempt to replace dead cells will need to utilize an external source. Stem cell-based therapies have been proposed for SCI based on their ability to meet many of these criteria, including modulation of the inhibitory microenvironment and replacement of cells lost through injury.

1.4 Existing stem cell sources for SCI therapy

The use of several stem cell sources for SCI therapy have been attempted with varying degrees of success, including non-neural lines. Mesenchymal stem cells have been shown to nullify the inhibitory micro-environment, resulting in increased endogenous regeneration (Oliveri et al., 2014). These treatments, however, did not produce new neurons to replace those lost through injury. The production of new neurons may well be required for full recovery. In addition, the consequences of introducing non-endogenous cell types to the spinal cord may not yet be fully understood. In an attempt to utilise a stem cell source less foreign to the spinal cord, transplantations of olfactory ensheathing glia (OEG), isolated from the olfactory bulb, have been tested in a variety of injury conditions. These have resulted in considerable motor and sensory improvements, both in rodent models and human trials (Tabakow et al., 2014). However, while these cells are surgically harvested from an isolated brain region, and the most probable side-effects of the

surgery (e.g. anosmia) may well be deemed an acceptable cost, brain surgery always carries risk of more severe consequences for the patient, already severely compromised with SCI. Neural stem cells (NSCs), taken from the subventricular or subgranular zones of the brain, or the ependymal canal of the spinal cord, are similarly difficult to harvest without significant risk to the patient (Poser and Androutsellis-Theotokis, 2013). Embryonic stem cells have been proposed for their ability to form all neuronal subtypes required. However, any transplantation of embryonic stem cells would almost certainly be allogeneic, with recipients likely requiring immunosuppression (Donnelly et al., 2012). Additionally, their sourcing is overshadowed by ethical issues, again marring success following transplantation (Lo et al., 2009). Finally, induced pluripotent stem cells (iPSC), while advantageous in many ways, carry considerable tumorigenic risk (Kuroda et al., 2013). Although this risk has been significantly reduced following several notable breakthroughs, other concerns regarding unknown consequences of epigenetic alterations during their generation (Lund et al., 2012) throws any near-future application of these cells into doubt.

Thus, a variety of stem cell sources have shown great potential towards achieving partial, if not complete, functional recovery following SCI. Indeed, despite their various limitations, several stem cell sources have progressed to clinical trials. These will be briefly discussed in the following section.

1.5 Existing stem cell-based clinical trials for SCI

A number of clinical trials have been approved and conducted to date, further highlighting the promise of stem cells as a therapeutic option for SCI. Work by Keirstead *et al* utilised embryonic stem cells (ESC) to address

demyelination occurring following SCI. Keirstead *et al* optimised protocols for derivation and expansion of ESCs and subsequent differentiation towards an oligodendrocyte lineage (Nistor et al., 2005). Transplantation of this population was seen to result in significant functional improvements in rat models of SCI (Faulkner and Keirstead, 2005; Keirstead et al., 2005). Importantly, axon myelination by OPCs following transplantation into *shiverer* mice (homozygous for mutations affecting the myelin basic protein gene) was demonstrated, showing both that transplanted OPCs differentiate towards a mature oligodendroglial lineage *in vivo* (via co-labelling with BrDU) and that they are able to fulfil their intended role. These results laid the foundation for progression to clinical trials.

A phase I clinical trial began in 2010, with brief hiatuses owing firstly to concerns over stem cell purity; secondly to fears surrounding possible cyst formation noted in the clinical trial data; and lastly to financial difficulties of Geron, of Menlo Park, California (Baker, 2011). The trial subsequently continued following acquisition of Geron by Asterias Biotherapeutics, also of Menlo Park (Hayden, 2014). The trial was concluded and reported safety of the transplanted cells, as well as possible cavity reduction, though no functional benefit (though cells were transplanted at a projected sub-clinical dose, so the lack of functional improvement is not unexpected (ClinicalTrials.gov, clinical trial identifier NCT01217008). Based on these data and additional supporting pre-clinical data (Priest et al., 2015), a phase I/IIa dose escalation study is currently underway (clinical trial identifier: NCT02302157). The difficulties faced throughout these studies raises an important point: despite the promising basic research supporting the use of this cell technology and the encouraging safety data obtained during the phase I trial, progression of these ESC-derived cells towards therapeutic application has been both difficult and incredibly expensive. This highlights

the importance of extensive preclinical data, however even this is no guarantee of advancement to the clinic.

While the above trial by Asterias was the first clinical trial to transplant differentiated cells derived from ESCs into humans (Hayden, 2014) it is no longer the only stem cell clinical trial aimed at demonstrating therapeutic potential of stem cells for SCI. Cummings *et al* showed the potential of human foetal brain-derived neural stem cells for SCI (Cummings et al., 2005). The authors noted appropriate neuroglial differentiation and long-term survival following transplantation into immune-compromised mice. Transplantation resulted in significant functional improvement, as assessed using BBB scoring. Significantly, these functional improvements were lost following selective ablation of transplanted cells. However, progression to the clinic was, in this case, hampered by advancement from research to clinical grade cells (Anderson et al., 2017). The authors attribute this discrepancy to variability in cell lines as well as inconsistencies following good manufacturing practice (GMP) scaling towards clinical quantities. Further, the authors point to the lack of FDA requirements for *in vivo* testing of the final clinical product prior to initiation of clinical trials (Anderson and Cummings, 2016). Despite this, StemCells Inc proceeded with a clinical trial testing the efficacy of a cell line based on the preceding results (clinical trial identifier NCT02136876) in 2014. However, while initial reports were positive, this trial was ended in 2016 citing a lack of significant improvements with time. These studies again highlight the difficulties in translating promising research from bench to bedside. Of key importance, these publications underline the need for critical evaluation of any given stem cell source at all stages progressing to the clinic, including basic research of stem cell efficacy and testing of the final product prior to clinical trial initiation. Indeed, the need to address consistency in cell products following scale-up of the initial

protocol has been recognised, particularly for generation of stable iPSC lines (Silva et al., 2015).

Despite the difficulties experienced it has been posited that progression of further basic research to clinical trials is vital for the development of the field (Fehlings and Vawda, 2011). Numerous clinical trials have demonstrated the safety of stem cell transplantation and some have shown slight functional improvements (Bunge, 2016; Dai et al., 2013; Park et al., 2012; Tabakow et al., 2013; Zhou et al., 2012). Importantly, in addition to providing vital safety data, these trials have helped refine human transplantation techniques. Clearly, any future progression from basic research to a clinical trial should proceed with caution, and of course not all laboratories have the necessary funding to support progression to, for example, large animal models (a highly desirable platform for testing prior to clinical trials). Nonetheless, with transparent presentation of findings, use of validated, and therefore comparable techniques in established models of SCI, and especially with careful evaluation of the final cell-based product before advancement, it could be argued that the field is ready for progression of more basic research to the clinic.

However, while many existing stem cell sources have been extensively researched, it is apparent from the existing literature that currently, no stem cell source represents an ideal solution. Despite this, the demand for an effective therapy is such that several imperfect sources have reached clinical trials, costing millions of pounds. Clearly, therefore, any potential new sources should be closely examined to determine whether they represent a significant advantage over existing ones.

1.6 Enteric neural system stem cells (ENSCs) as a potential source of stem cells for SCI

Enteric neural stem cells (ENSCs), isolated from the adult gut, are a promising alternative stem cell source. These are the resident stem cells of the ENS, and have been isolated from patients up to 80 years of age (Metzger et al., 2009a). They are derived from neural crest cells (NCCs) - a transient population of cells that give rise to numerous cell types throughout the body including the ENS of the gastrointestinal tract (Yntema and Hammond, 1954), and transplantation of these cells into the gastrointestinal tract shows that they have the ability to differentiate into neurons and glial cells (reviewed in Burns and Thapar, 2014). Furthermore, following transplantation these neurons have been shown to integrate functionally into the endogenous ENS networks (Burns and Douarin, 1998; Cooper et al., 2017), and even to rescue a loss of GI function (McCann et al., 2017). Recently ENSCs cultured from postnatal human tissue have been shown to retain similar migratory, proliferative and differentiation capabilities to embryonic neural crest-derived cells, demonstrating their potential benefits towards tissue regeneration following transplantation (Cheng et al., 2017)

1.6.1.1 Enteric Nervous System Development

The ENS is arguably the most complex division of the PNS, containing more neurons than the spinal cord (Furness, 2006). This cell density is matched by the diversity of neuronal subtypes and range of neurotransmitters produced (McConalogue and Furness, 1994; McConalogue et al., 1994). The neurons and glia of the ENS form an intricate network responsible for the peristaltic propulsion of boluses along the GI tract, enzymatic secretion, nutrient and

water absorption, and regulation of blood flow within the intestinal wall, among other functions (Furness, 2006). Indeed, it has been shown that many of these processes occur with partial or even complete autonomy from the CNS. When CNS innervation of the GI tract is severed, many regions of the GI system are entirely capable of sustaining normal functions, demonstrating the sophistication of the ENS neural network (Bayliss and Starling, 1899). The ENS is organised into and mediates this intrinsic control through two concentric plexuses (the myenteric and submucosal) spanning the entire length of the GI tract (Furness, 2012). These neural plexuses are embedded within the complex laminated structure of the adult gut wall (Fig. 1.4).

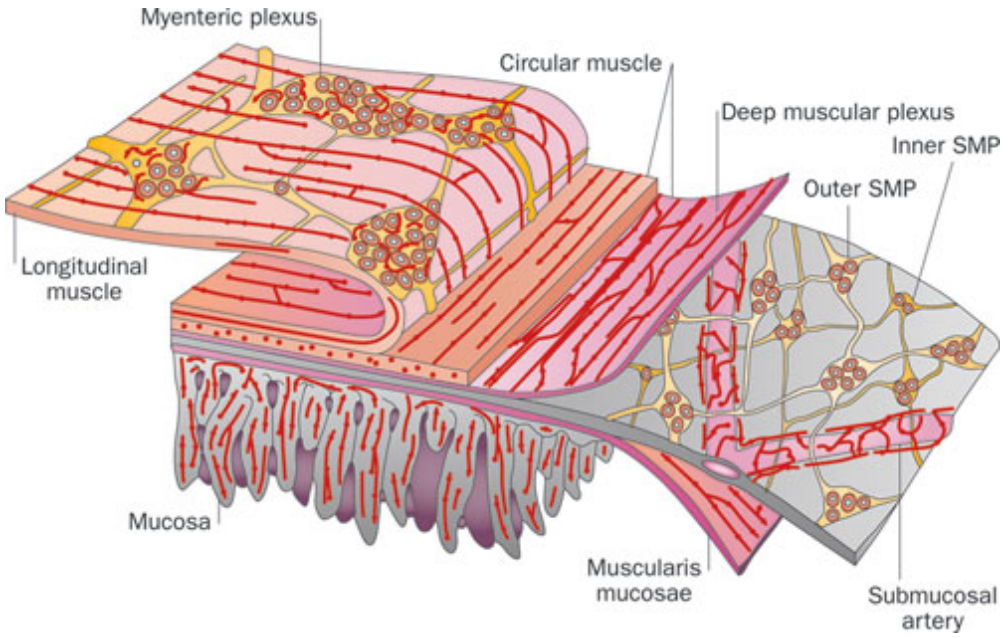

Figure 1.4: The enteric nervous system consists of two concentric plexuses, forming interconnected neuronal networks.

The myenteric plexus (located between the longitudinal and circular muscle layers) and the submucosal plexus (located between the circular muscle and muscularis mucosa) innervate the gastrointestinal tract. Taken from (Furness, 2012).

The ENS is derived in its entirety from NCCs that migrate from the dorsal neural tube early in development (Gershon et al., 1993; Yntema and Hammond, 1954). The neural crest comprises a highly migratory, multipotent cell population that gives rise to multiple cell types, including mesenchymal lineages such as adrenal medulla tissue, craniofacial structures including dentine of teeth, melanocytes, cardiac regions, and neuroectodermal lineages such as the peripheral nervous system (Le Douarin and Smith, 1988; Le Lievre and Le Douarin, 1975; Miletich and Sharpe, 2004). The incredibly diverse fates of NCCs has led many to refer to these cells as the fourth germ layer (Shyamala et al., 2015).

The fate of NCC is at least partially determined prior to leaving the neural tube by their relative positioning along the anterior-posterior axis of the developing embryo. NCCs are formed along the entirety of the neural tube, and prior to their migration neural crest regions can be divided both anatomically and functionally into cranial, vagal, cardiac, trunk and sacral populations (Vaglia and Hall, 1999). The vast majority of the ENS is formed from vagal neural crest, with modest contributions from the sacral population (Burns and Douarin, 1998) (Fig. 1.5).

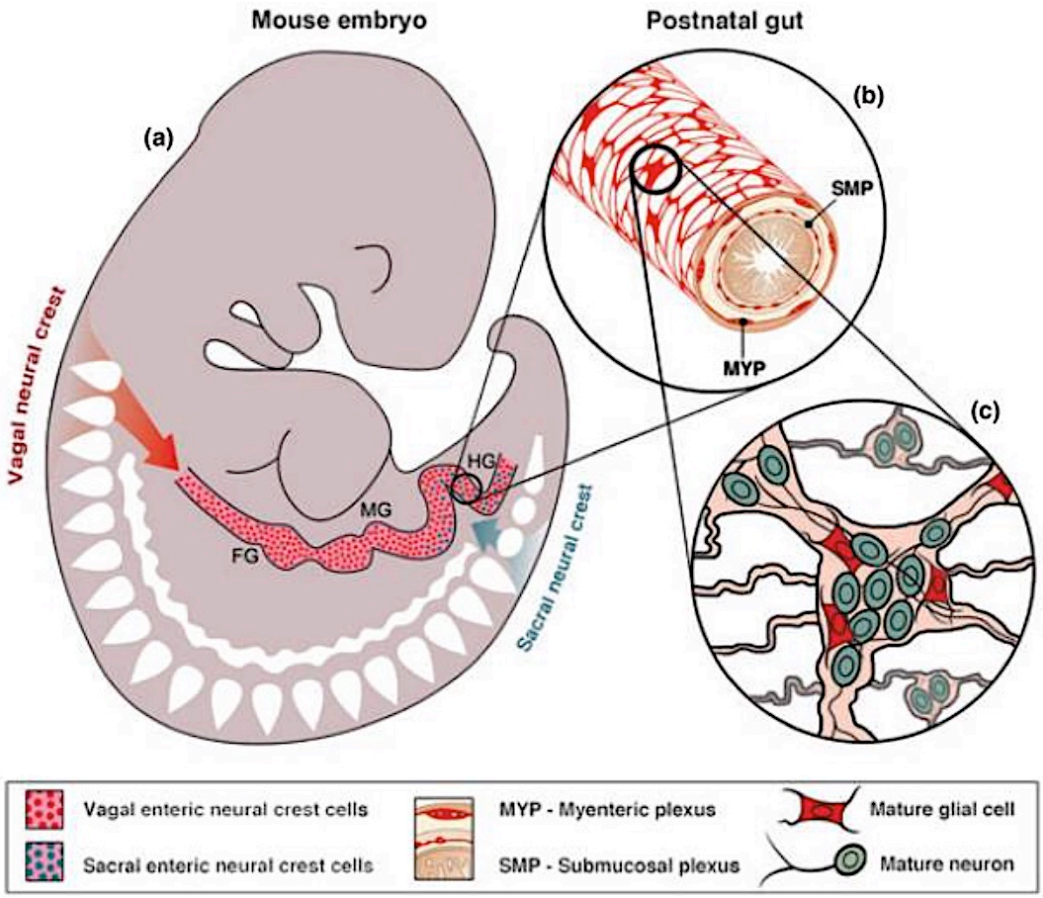

Figure 1.5: The enteric nervous system is derived from the vagal and sacral neural crest.

The neural crest, formed at the boundary between the neural tube and the overlying ectoderm, migrates from the dorsal neutral tube to the developing gut. The vagal neural crest (adjacent to somites 1-7) migrates first, reaching the foregut and migrating caudally throughout the gut to form both the myenteric and submucosal plexuses. The sacral neural crest (beginning caudal to the 28th pair of somites) enters the gut once colonization of the entire GI tract by the vagal neural crest is essentially complete, and thence contributes only to the formation of hindgut ENS. Once complete, the ENS consists of two concentric structures; the myenteric and submucosal plexuses. These are formed of interconnected ganglia composed of neurons, glia and endogenous stem cells. Taken from (Goldstein et al., 2013).

Generally, vagal NCCs migrate to and enter the developing GI tract at the level of the foregut. In humans this initial invasion takes place around 4 weeks into gestation (Heanue and Pachnis, 2007; Wallace and Burns, 2005). The cells then migrate in a rostral-caudal direction, colonising the entire length of the gut, a process taking around 3 weeks in humans (Anderson et al., 2006; Wallace and Burns, 2005). This migration is then followed by differentiation and maturation of ENS structures occurring in a similar rostrocaudal sequence. Proximal to the vagal migration wavefront, NCC progenitors stop migrating, proliferate and differentiate to form ganglia - interconnected clusters of several neuronal subtypes and glia. These ganglia further interconnect to ultimately form the myenteric and submucosal plexuses, comprising the functional neural circuits that regulate GI function. In both mammals and birds, the sacral NCC contribution is restricted to the distal hindgut, where the pioneer cells initially migrate to regions of close proximity to the hindgut – the avian-specific nerve of Remak in the chick (Burns and Le Douarin, 1998), and the pelvic plexus in mice (Anderson et al., 2006; Wang et al., 2011). They then pause until vagal colonisation of the hindgut is complete before entering the gut to produce neurons and glia. In humans, vagal colonization is complete after around 7 weeks of gestation (Wallace and Burns, 2005), reviewed in (Heanue and Pachnis, 2007). Up to 17% of myenteric ganglia neurons are sacral-derived in the chick, with far fewer cells found in the submucosal plexus (Burns and Douarin, 1998). In mouse the sacral input has not been quantified, but appears similar (Wang et al., 2011).

1.6.1.2 Suitability of enteric neural stem cells for spinal cord injury therapy

The substantial proliferative capacity of enteric neural crest cells (ENCCs), as demonstrated by the formation of such an extensive and diverse structure as the ENS from a relatively small number of initiating cells, almost certainly persists to a certain degree into adulthood. Indeed, cells with stem cell-like capability – ENSCs - have been isolated from patients as old as 84 years (Metzger et al., 2009a), and have been expanded *in vitro* to form neurospheres. In the Burns/Thapar laboratory, ENSCs have been isolated from human gut biopsies and expanded *in vitro* to form neurospheres (Metzger et al., 2009b). Similar neurospheres have also been generated from ENSCs isolated from mice, and have been extensively characterised by immunofluorescence and qRT-PCR, revealing the presence of neurons, glia and stem cells (Binder et al., 2015; McCann et al., 2017). ENSC neurospheres are capable of restoring ENS structures and producing neurons and glia when transplanted into aganglionic mouse gut *ex vivo* (Natarajan et al., 1999), reviewed in (Burns and Thapar, 2014; Heanue and Pachnis, 2007). Even more encouragingly, ENSCs transplanted *in vivo* have been shown to form functional connections with the endogenous network (Belkind-Gerson et al., 2016; Cooper et al., 2016; Cooper et al., 2017), and to rescue normal gut function in a mouse model of enteric neuropathy (McCann et al., 2017).

The presence of a stem cell population in the adult ENS offers a promising source for stem cell-based therapies for SCI. Not only is the ENS known to share many similarities with the CNS, and thus is a desirable source for stem cell-based SCI therapy, it can also be repeatedly accessed via routine endoscopy to generate an autologous cell source for transplant, thus avoiding the harvesting/ethical issues that hinder other NSC sources. Such utilisation of the ENS stem cell pool is already being pursued for enteric

neuropathies and has demonstrated considerable early success (Burns and Thapar, 2014; McCann et al., 2017). The goal of this thesis was to explore whether stem cells derived from the ENS could also be used for the treatment of CNS insults such as SCI.

1.6.1.3 Physical connections between the enteric and central nervous systems

When evaluating stem cell sources for SCI therapies, it would be advantageous if donor cells were capable of producing cells that had the ability to integrate and communicate with existing spinal cord circuits. ENSCs are likely able to fulfill this criteria for a number of reasons. The ENS and CNS are physically connected and in constant communication through the gut-brain axis (Furness, 2012). While the ENS conducts most of its functions independently of CNS intervention (Bayliss and Starling, 1899), the two are intimately linked to allow CNS modulation of ENS activities and transfer of information between the two systems (Browning et al., 2017) (Fig. 1.6). Higher brain centres, such as the basal ganglia, exert control through either vagal pathways (usually for oesophageal targets); sympathetic pathways acting on local reflex loops of the myenteric and submucosal plexus; or through pelvic pathways (usually for colon targets). Communication is bidirectional, with ENS axons projecting to the spinal cord (Furness, 2012). This axis affects brain development (partially through the gut-brain-microbiota axis (Sherwin et al., 2016), and has even been implicated in the initiation/development of neurodegenerative disorders (Mulak and Bonaz, 2015). This close interaction and information transfer strongly suggests that, if ENSCs were utilized for SCI therapy, transplanted cells would be able to integrate with endogenous spinal cord neurons to restore function.

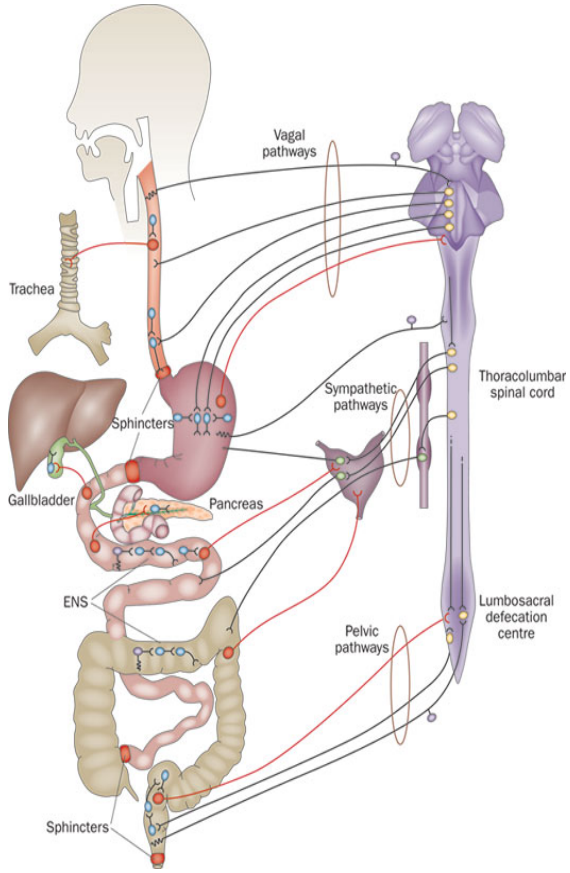

Figure 1.6: The enteric and central nervous systems are intimately connected.

Several pathways extend from the spinal cord to integrate with the ENS either directly (yellow), or via prevertebral ganglia (green). The degree of CNS input varies along the GI tract. The oesophagus and anus are almost entirely under central control, while the small intestine receives little input. Communication is bidirectional, with neurons from the intestines (red) projecting back to the spinal cord. The ENS also contains multiple reflex circuits (blue), allowing it to function largely independently of CNS control. Taken from (Furness, 2012).

1.6.1.4 Cellular composition of the enteric and central nervous systems

Given their shared ectodermal origin and numerous physical connections, it is not surprising that the ENS and CNS also contain similar cell complements. Glia comprise the majority of cells within the CNS, including astrocytes, oligodendrocytes and microglia (Gonzalez-Perez et al., 2015). CNS astrocytes share almost all properties and characteristics of enteric glia of the ENS (Gershon and Rothman, 1991; Gulbransen and Sharkey, 2012), but other glial populations are more distinct. Oligodendrocytes are responsible for

myelination in the spinal cord, but such insulation does not occur in the ENS (Bradl and Lassmann, 2010; Ruhl, 2006). NCCs are capable of producing myelinating cells - PNS Schwann cells are derived from NCCs and are known to invade the gut during development, but only contribute towards neuronal lineages (Uesaka et al., 2015). Presumably, the lack of myelination is attributable to the relatively short distance ENS axons are required to project.

Microglia are the resident immune cells of the spinal cord, with phagocytic clearance roles. In the ENS this responsibility is assumed by macrophages (Mikkelsen and Rumessen, 1992; Mikkelsen et al., 1985). Macrophages are found throughout the body, and assume variable phenotypes depending on their localisation. However, significant similarities between CNS microglia and ENS macrophages have been highlighted. Indeed the two are difficult to distinguish, with a common myeloid progenitor, similar morphology and shared surface markers (Bain et al., 2014; Bain and Mowat, 2014; Geissmann et al., 2010a; Geissmann et al., 2010b; Ginhoux et al., 2010).

Slight cellular discrepancies may prove beneficial for SCI cell-based therapies. While astrocytes are fundamental to limiting lesion spread, their assimilation into the glial scar prevents axonal regeneration (Sun and Jakobs, 2012). While enteric glia are remarkably similar to CNS astrocytes, it is not known whether enteric glia would contribute to or limit the astrocytic aggregation resulting in formation of the glial scar. The lack of myelinating cells within the ENS is regrettable for treatment of SCI, but a de-differentiation of the Schwann cell-derived neurons is entirely plausible, presenting a potential source of myelinating cells. Additionally, it has been demonstrated that following SCI and the subsequent demyelination, there is an invasion of re-myelinating Schwann cells from the PNS (Guest et al., 2005;

James et al., 2011). Therefore, stimulation to encourage further Schwann invasion and increased endogenous re-myelination may remove the need for transplanted cells to contain myelinating cells.

Like their respective glial populations, the neuronal complement of the ENS and CNS is also similar, with only slight variations in relative quantities of neuronal subtypes between the two systems. In the spinal cord, motor neurons compose the majority of cells within the grey matter, alongside large numbers of interneurons (while axons of sensory neurons project extensively throughout the cord, their cell bodies are located in peripheral tissues, and often synapse at the dorsal root ganglia, where interneurons continue the pathway) (Caspary and Anderson, 2003; Gouti et al., 2015). The ENS contains a more varied array of motor, sensory and interneurons to allow it to function with autonomy (Costa et al., 2000a; Costa et al., 2000b; Furness, 2000). This multiplicity of lineages will likely prove beneficial in restoring spinal circuits, affording flexibility of transplanted ENSCs to provide required cell types. For example, relay circuits may be formed between spinal ganglia-derived sensory axons entering the SC, and transplant-derived enteric sensory neurons within the injury zone, to re-establish ascending sensory pathways.

1.6.1.5 Neurotransmitters in the enteric and central nervous systems

Although transplanted mesenchymal stem cells have resulted in significant functional recovery following SCI without neurotransmitter production (Oliveri et al., 2014), an ideal cell source for SCI would produce the appropriate cells types able to synthesise neurotransmitters to allow continuous communication through the injured region. Indeed, several

neurotransmitters have been shown to be of vital importance following SCI (several of the most prominent are summarised in Table 1.1).

The ENS utilises almost all of the same neurotransmitters as the CNS (McConalogue and Furness, 1994). This complex signalling network is reflective of the diverse range of GI functions, including modulating peristalsis and responding to the microbiome within the bowel. While some bowel actions are CNS instigated (e.g., stomach churning directed by the vagal nerve) many of the feedback loops responsible for bringing about such changes begin and end in the ENS, requiring a diverse and sophisticated communication system (Furness, 2012).

Table 1.1: Neurotransmitters noted as particularly important following spinal cord injury and their roles in the enteric and central nervous systems.

Neuro-transmitter	First discovered	Role in ENS	Role in CNS	Role in SCI
Acetylcholine (ACh)	1934 -(CNS) (Dikshit, 1934) 1937 -(ENS) (Dale, 1937)	Major excitatory neurotransmitter, gut motility (Borody et al., 1985), homeostatic ion control (Frieling et al., 1992), entero-endocrine release (Chey	Sensory/ motor transmission, (Bulbring and Burn, 1941; Myslinski and Randic,	Contributes to muscle spasming (Kapitza et al., 2012)

		and Gutierrez, 1978)	1977) nocicepti ve signalling (Zhuo and Gebhart, 1991)	
Serotonin (5-HT)	1953 - (CNS) (Twarog and Page, 1953) 1937 - (ENS) (Vialli and Erspamer, 1937)	Peristaltic reflex and modulation of gut motility (Bulbring and Crema, 1958; Bulbring and Lin, 1958)	Neuro-modulati on of motor and sensory function (Jacobs and Fornal, 1997; Jacobs et al., 2002; Loyd et al., 2013)	Muscle paralysis, motor neuron recovery (Murray et al., 2010)
Gamma amino butyric acid (GABA)	1950 -(CNS) (Roberts and Frankel, 1950) 1979 -(ENS)	Excitatory (neuronal), inhibitory (enteroendocrine cells), mediates	Major inhibitory neurotran smitter, nocicepti	Hyperexcit-ability of surviving cells (Gwak et al.,

	(Jessen et al., 1979)	neuroimmune interactions (Auteri et al., 2015a, b)	ve signal modulation (Guo and Hu, 2014)	2012), neuropathic pain (Gwak et al., 2006)
Nitric oxide (NO)	1988 -(CNS) (Garthwaite et al., 1988) 1990 - (ENS) (Bult et al., 1990)	Inhibitory motor neurotransmitter (Nezami and Srinivasan, 2010)	Inhibitory motor neurotransmitter	Inflamm-ation during the secondary injury phase (Hamada et al., 1996; Matsuyama et al., 1998)
Glutamate	1952 -(CNS) (Curtis et al., 1960; Martinez-Hernandez et al., 1977) 1986 -(ENS) (Jessen et al., 1983)	Largely unknown	Major excitatory neurotransmitter (Kew and Kemp, 2005)	Contributes to excito-toxicity in SCI (Park et al., 2004)

1.7 Summary

Injury to the spinal cord results in a complex cascade of secondary mechanisms leading to the formation of fluid-filled cavities. These cavities

contain a variety of inhibitory factors, hindering endogenous regeneration. In addition, the spinal cord is unable to replace lost cells or restore severed axonal pathways. Any therapy targeted towards restoring spinal cord function after SCI must address these issues. Currently, all stem cell sources that have been tested for SCI therapy have proven unsatisfactory, leaving an urgent need to explore other options. ENSCs isolated from the ENS may fulfill this need. The ENS and CNS share many similarities in terms of origin, cell complement and neurotransmitter production. Indeed, the potential of ENSCs for SCI was first recognized as far back as 1993 (Jaeger et al., 1993). Previous studies in the Burns/ Thapar laboratory have optimized techniques to harvest, expand and characterise ENSCs, revealed the colonizing ability of these cells when transplanted into aganglionic or dysfunctional gut, and shown their capacity to functionally integrate into existing circuitry. Taken together, this suggests the strong potential of ENSCs to serve as a viable stem cell source for SCI therapy.

1.8 Thesis aims:

Published observations in the literature demonstrate numerous similarities between the enteric and central nervous systems, and the ability of the two to communicate. Considering the multitude of ethical and practical issues surrounding harvesting and/or transplantation of previously proposed stem cell sources for SCI, an assessment of the biology and therapeutic potential of ENSCs for SCI is both appropriate and necessary.

1. The first aim of this thesis is to provide proof of principle data that ENSCs can survive in a CNS environment (SC). This was achieved via chimeric grafting of GFP/WT chick embryos to label NCC-derived cells, and transplantations of GFP+ enteric, ENSC-containing neurospheres into the developing chick neural tube. These results are presented and discussed in chapter 2.
2. The second aim of this thesis was to progress these embryonic studies into an adult rodent model of SCI. A pilot study was conducted to determine optimal time post-injury of transplantation into a rat spinal cord contusion model, and to assess the short-term survival of transplanted, rat-derived enteric cells (including ENSCs) in an injured spinal cord environment. These results are presented and discussed in chapter 3.
3. The third aim of this thesis was to conduct an extended study into the survival, differentiation and effect of ENSCs both on the spinal cord lesion histology, including endogenous recovery, and on functional recovery post-injury. Simultaneously, the benefits of a combinatorial application of chondroitinase ABC (ChABC) and transplanted enteric

cells was assessed. These results are presented and discussed in chapter 4.

4. Finally, the overall results of the thesis, particularly as relates to the existing literature, and the future direction of both this work and research into SCI in general, are discussed in chapter 5.

Chapter two: Engraftment and Survival of Enteric Nervous System-derived Stem Cells in the Regeneration-Permissive Chicken Embryo Spinal Cord

2.1 Introduction

While considerable evidence supports the potential compatibility of ENS-derived cells with the endogenous cells of the CNS, to our knowledge transplantation of ENSCs into the injured spinal cord is entirely untested (a previous publication tested SCI transplantation of intact nervous tissue taken from the gut, but not isolated stem cells (Jaeger et al., 1993)). The ideal way to test the ability of ENSCs to survive in a CNS environment would be an *in vivo* transplant. In order to determine the likely success of ENSC transplantation into injured spinal cord, the chicken embryo was utilized as a practically feasible model to provide proof of principle data.

Historically the chicken has been a vital tool for scientific research, and may well be the oldest model organism studied. Its embryonic development was first analysed by Aristotle in his work *Historia Animalum*, and variations in chicken breeds were later used as examples by Darwin in *The Variation of Plant and Animals under Domestication*, in which he set out his theories of evolution. Apart from being inexpensive, there are a number of features that make the chicken an advantageous model. Perhaps most importantly, unlike most other vertebrate species the live chicken embryo can be easily observed by the creation of a small window in the shell of a fertilized egg, allowing its real-time development to be studied *in ovo*. Due largely to this ease of accessibility, the chicken proved indispensible for early studies concerning embryology (Fig. 2.1). Much of the fundamental knowledge of vertebrate embryonic development is based on studies conducted in chicken embryos, considerable amounts of which are directly comparable to human development through conserved evolutionary pathways (Burt, 2007; Stern, 2005).

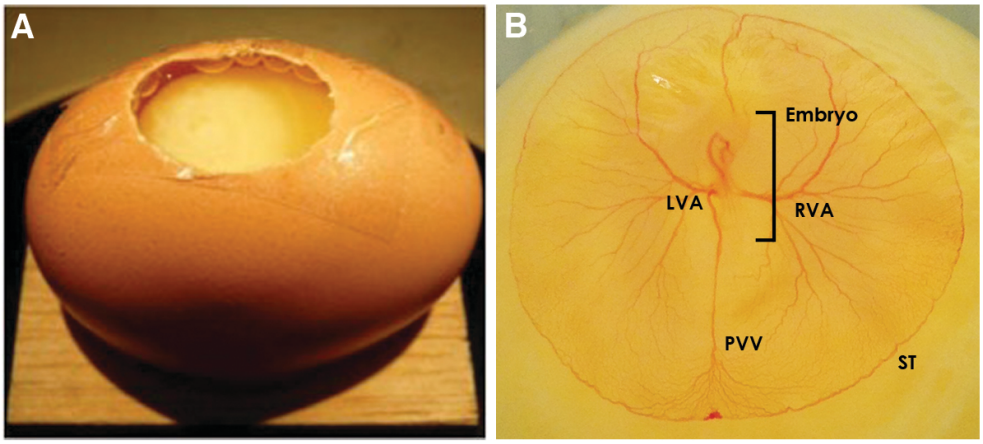

Figure 2.1: Chicken embryos can be accessed and manipulated *in ovo*.

The chicken embryo is one of the oldest model organisms for the study of developmental biology. Through the creation of 'windows' in the shell (A) the embryo can be accessed, allowing, among other procedures, chimeric grafting to label NCCs. At E3 the embryo lies on top of the yolk with an extensive surrounding vasculature system (B). LVA – left vitelline artery, RVA – right vitelline artery, PVV – posterior vitelline vein, ST – sinus terminalis.

Initial experiments were based simply on observation, which in itself yielded fruitful information such as the elucidation of the existence of the blastocyst and germ layers (Pander, 1817), and the development of the circulatory system (Harvey, 1628). The chicken embryo was found to be robust enough to withstand manipulation, allowing for the ablation of specific tissues and observation of the subsequent defects. As techniques grew more sophisticated, ablations were supplemented with 'grafting', in which parts of one embryo (e.g. neural tube) were removed, manipulated (for instance, by inversion) and re-inserted. These studies yielded fundamental understanding of patterning during embryogenesis, determining the importance of the order of tissue formation (Saunders, 1948) and the vital

role of organisers such as Hensen's node (Waddington, 1950). Many of these studies informed current medical knowledge and surgical techniques. It was the grafting of various tissues into the chicken embryo that revealed the importance of the immune system in the graft *vs* host response (Murphy, 1914), a key finding which informed human organ transplantation and a multitude of other surgical interventions.

These procedures were further refined in pioneering work by Nicole Le Douarin, who demonstrated that tissues could be transplanted from a quail embryo into a chicken embryo, and vice versa, and the cells of the two species distinguished histologically (Le Douarin, 1973). This allowed more precise tracking of the consequences of grafting through interrogation with species-specific antibodies. These experiments provided unprecedented information concerning specific migratory pathways and, in particular, greatly expanded the existing knowledge of neural crest and its migration to form the ENS. In a series of landmark experiments, Le Douarin and others showed in exquisite detail, via grafting of the neural tube from a quail into the corresponding region of a chicken embryo, the ventral migratory pathways taken by the neural crest to enter and colonize the developing gut (Burns and Douarin, 1998; Le Douarin and Teillet, 1973).

Recent advances in the field of genetics have yielded transgenic chickens modified to express green fluorescent protein (GFP) under the control of a ubiquitous promoter (McGrew et al., 2004). This breakthrough allowed intra-species grafting between GFP-expressing chick donor tissue and WT chick hosts, negating the often-cited possibility that grafted chicken cells behaved differently in the host quail tissues than they would have done in their native environment. Using such techniques, fate maps for the various neural crest populations have been precisely documented, revealing the exact neural crest contributions to structures such as the lungs (Freem et al.,

2012).

Perhaps due in part to the rich wealth of preceding data, the chicken embryo continues to serve as a model organism not just for developmental research, but for disease and even injury/ regenerative research, including SCI (Prasongchean et al., 2012). The developing chicken neural tube can be easily accessed and manipulated at various stages of development, allowing identification of key developmental periods. In this way it was demonstrated that before embryonic day (E) 13, the chicken spinal cord is entirely capable of regeneration following injury, and that after this point, injury results in varying degrees of paralysis (Hasan et al., 1991; Shimizu et al., 1990). Keirstead *et al* noticed that the end of the regeneration permissive phase coincided with the onset of myelin production. These authors showed that delaying myelin production in the developing chick embryo resulted in an extension of the 'regeneration permissive' phase, providing compelling evidence for the inhibitory role of myelin in spinal cord injury recovery (Keirstead et al., 1992).

Thus, the chicken embryo serves as an ideal model with which to begin studies into the therapeutic potential of ENSCs for SCI repair. Given that the immune system does not develop in the chicken embryo until around E12 (and indeed, is still immature at birth) (Moticka, 1975), this allows ENSC transplantation into the spinal cord to be tested without the immune response associated with SCI in adults, thus providing vital proof of principle data that ENSCs are capable of surviving in a spinal cord environment.

2.1.1 Chapter aims and objectives

The aim of this chapter is to evaluate the potential of stem cells derived from the enteric nervous system as a therapy for SCI, using the chick as a model organism. This will be addressed by assessing the ability of ENSCs

transplanted into the spinal cord to survive, differentiate into appropriate cell types, and bridge the injury zone in the embryonic spinal cord.

Objectives:

1. Label NCC derivatives, including ENSCs, within the gut of chick embryos by grafting the vagal neural tube of GFP chick embryos into WT age matched chick embryos.
2. Isolate labeled cells using fluorescent activated cell sorting (FACS) and culture to form neurospheres.
3. Characterize isolated cells, including determination of neurosphere cell composition.
4. Transplant labeled neurospheres into early chick embryo spinal cords and assess cell survival and differentiation.
5. Transplant labeled neurospheres into late stage chick embryo spinal cords and assess survival and differentiation.

2.2 Methods

All experiments conducted on chicken embryos were completed before three quarters of the way through embryo development (i.e. before E15) and therefore did not fall under the UK Animal (Scientific Procedures) Act.

2.2.1 Preparation of chicken embryos for surgical manipulation

Fertilised wild type (WT) chicken eggs, obtained from commercial sources (Medeggs, UK) were kept in a humidified incubator at 37°C for 36 hours, to embryonic day (E) 1.5. A small hole was made at the pointed end of each egg and 2mL albumin extracted using a 32-gauge needle (lowering the embryo away from the shell surface and preventing embryo damage). This hole was sealed using clear tape, and another hole made in the upper egg surface. Curved scissors were used to make a 2.5cm diameter window on top of the egg. The window was sealed using clear tape and eggs returned to the incubator.

2.2.2 GFP labelling of neural crest cells using tissue grafting

Host tissue.

To visualise windowed WT embryos at E1.5, a small amount of Indian ink, diluted 1:1 with 1Xphosphate-buffered saline (PBS) with penicillin/ streptomycin (PS) was injected under the embryo of windowed eggs using a mouth pipette and pulled glass needle. The vitelline membrane was torn to expose the region of interest and the vagal region of the neural tube (somites 1-7) removed using a custom made micro-scalpel. When performed correctly, this ablation leaves surrounding tissue intact (Fig. 2.1).

Donor tissue.

Fertilised GFP chicken eggs were obtained from The Roslin Institute Transgenic Chicken Facility, Scotland, UK, and incubated to E1.5. GFP+ eggs were windowed as above, and the embryo harvested and transferred to a sylgard-based watch-glass filled with 1XPBS with penicillin/ streptomycin. The embryo was separated from the vitelline membrane and pinned using minutien pins. The vagal region of the neural tube was isolated using micro scissors and transferred to a watch-glass filled with 2% pancreatin (Sigma Aldrich, UK) in 1XPBS with penicillin/ streptomycin at RT. After 8 minutes the neural tube was teased from the surrounding structures using micro pins and transferred to DMEM+10% normal sheep serum to halt enzymatic digestion.

Grafting procedure.

Grafting procedures were conducted at room temperature (RT). The harvested GFP+ neural tube was transferred to the host WT egg using a mouth pipette and pulled glass needle, and positioned along the correct anterior-posterior axis. The neural tube was grafted into the ablated region via manipulation with micro-needles, and fluid surrounding the embryo was drained using a mouth-pipette to aid graft adhesion. The egg shell window was re-sealed and the eggs containing chimeras returned to the humidified incubated at 37°C.

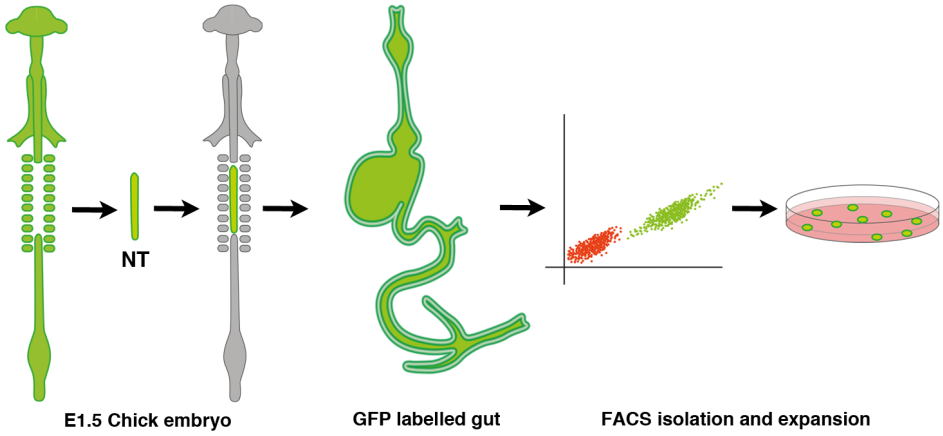

Figure 2.2: GFP+ neural tube tissue from E1.5 donors was grafted into age-matched WT hosts to label neural crest-derived cells.

The vagal neural tube (located between somites 1-7) of GFP+ embryos was isolated and grafted into WT hosts with the corresponding region ablated. Embryos were returned to the incubator for 12.5 days, at which point the intestines were harvested, stripped of mesentery, and dissociated into a single cell suspension. GFP+ cells were isolated by FACS and expanded in culture to form GFP+ neurospheres.

2.2.3 Cell culture and FACS analysis

FACS (fluorescence activated cell sorting) was used to isolate GFP+ neural crest-derived cells from GFP/ WT chimeras (Fig. 2.2). At E14 embryos were sacrificed, the intestinal tract harvested in ice-cold 1XPBS (Mg^{2+}/ Ca^{2+} free) with penicillin/ streptomycin (Sigma Aldrich, UK), and manually chopped into pieces ~2mm in length. These were washed in 1XPBS and digested in 1mL dispase/ collagenase (1mg mL^{-1}) (Roche, UK) at 37°C for 10 minutes. NSM+P (DMEM F12 (Sigma Aldrich, UK), N2, B27 (Invitrogen, UK), P/S with 2% foetal

bovine serum (FBS, Sigma Aldrich, UK)) was added to halt the reaction. The solution was centrifuged, re-suspended in NSM+P and passed through 40μm cell strainers. Cells were sorted based on GFP fluorescence using a MoFlow XDP (Beckman Coulter). Collected cells were plated onto 2% fibronectin (Sigma Aldrich, UK) coated plates. The following day the media was replaced to remove dead cells.

If colonies grew to confluence they were passaged. This involved a wash in 1XPBS and trypsin (Sigma Aldrich, UK) digestion for 2 minutes at 37°C. Cells were dissociated, centrifuged and re-plated onto fresh 2% fibronectin-coated dishes at the required density.

2.2.4 Viral labelling of cells

Chick spinal cord-derived cells were labelled with an mCherry lentivirus as previously published (Natarajan et al., 2014) prior to co-culture experiments with GFP-labelled ENSCs. The lentivirus used was a self-inactivating, HIV-1 based vector, containing the spleen focus forming virus LTR promoter. The mutated Woodchuck posttranscriptional Regulatory Element was inserted downstream of mCherry (Demaison et al., 2002). Flow cytometry was used to tire the virus. Cells were incubated with 100μL per 10^5 cells (multiplicity of infection 2-5) of the lentiviral construct diluted in NSM+P, for 48 hours to allow efficient transduction and viral inactivation. Following this, media was drained and treated with Virkon to destroy remaining viral particles. Cells were fed using fresh NSM+P.

2.2.5 Transplantation of GFP+ ENSC-derived neurospheres into early stage chick embryos

WT embryos for transplantation were incubated to E1.5, windowed and visualised. The vitelline membrane was torn to expose the region of interest. A micro-scalpel was used to remove a region of the neural tube equivalent to the length of 1 somite, at the level of somite 7 (this graft location reproducibly ensured transplants could be found in the cervical region of the spinal cord upon harvest). A GFP+ ENSC-derived neurosphere was transplanted into the ablated space. Excess fluid was removed to encourage adhesion. The window was re-sealed and the egg returned to the humidified incubator at 37°C (Fig. 2.3). Transplanted embryos were harvested at timed intervals (4, 6, 9 and 12 days after transplantation, to provide timed intervals up to ~¾ of chicken embryo development, after which the UK Animal (Scientific Procedures) Act) applied.

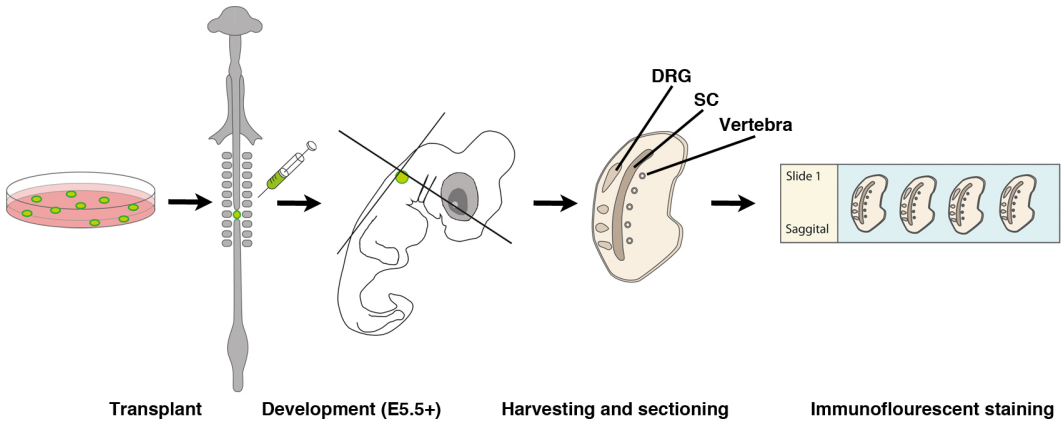

Figure 2.3: GFP+ ENSC-derived neurospheres were transplanted into early stage chick embryo neural tubes to determine survival.

At E1.5, WT embryos had a short region of the neural tube, equivalent to the length of one somite, removed at the level of somite 7. A GFP+ ENSC-derived neurosphere was transplanted into the ablated region, and the embryo returned to the incubator. Transplanted embryos were then harvested at timed intervals, and the tissue sectioned (coronal, saggital and longitudinal sections were utilised for this study) and stained for analysis.

2.2.6 *Ex ovo* cultures

Classic windowing techniques offer poor access to the spinal cord of late-stage embryos due to the embryo turning onto its side. To increase accessibility of embryos at 11 days of development, embryos were grown as *ex ovo* cultures. 8oz plastic cups were filled ¾ with distilled water. Saran wrap was placed on top and pushed to contact the water. An elastic band held the wrap in place. Eggs incubated to E3 were cracked and the embryo transferred gently onto the saran hammock. 1mL 1XPBS with penicillin/ streptomycin and crushed eggshell (a calcium source) were added. An 8cm

culture dish with ventilation holes was placed on top. The culture was placed in a humidified incubator at 37°C until required.

2.2.7 Inducing lesions and transplantation of GFP+ neurospheres into late stage chick embryos

Ex ovo injuries were induced under a stereo-microscope. The embryo was raised to the surface of the cup by tightening the hammock. Fine forceps (Dumont 5) were used to tear the vitelline and chorioallantoic membrane (selecting an area with few blood vessels) above the cervical spinal column. A curved, blunt glass rod was inserted under the neck and the embryo gently lifted to the surface. Forceps restricted to open to a maximum width of 1mm and marked to indicate a depth of ~2mm, were inserted through the dermis into the dorsal side of the spinal column in the cervical region. The forceps were fully closed for 5 seconds, opened and retracted. If required, a neurosphere was introduced into the injury site via a pulled glass needle. The injury site was observed for ~20 seconds to ensure the neurosphere did not float out. The glass hook was removed and the hammock lowered back into the cup. The embryo was returned to the humidified incubator at 37°C.

2.2.8 Gelatin embedding for sectioning

Harvested samples were fixed for 1 hour at RT in 4% paraformaldehyde (PFA, Sigma Aldrich, UK) and washed in 1XPBS. If tissue was to be used for whole mount staining no further procedures were necessary. If required for sectioning, samples were dehydrated in sucrose solution (30% sucrose in 1XPBS) overnight at 4°C and transferred to molten gelatin (15% sucrose,

7.5% gelatin in 1XPBS, Sigma Aldrich, UK) for 1 hour at 37°C. Samples were cast in gelatin blocks for longitudinal/ coronal sectioning and snap frozen in -65°C iso-pentane. Blocks were stored at -80°C.

2.2.9 Cryosectioning

Frozen gelatin-embedded samples were sectioned using a Leica Cryostat at -22°C. Section thickness was 10-20µm. Slides were stored at -20°C until required.

2.2.10 H&E staining

H&E staining was used to visualise histological aspects of spinal cord injury. Frozen slides were thawed for 40 min (RT) and immersed in pre-filtered Haematoxylin solution (Bayers, UK) for 7 minutes. Slides were washed in ddH_2O for 10 seconds, and running water for five min. Slides were dipped in acid alcohol (1% HCl in 70% ethanol) for 10 seconds and washed for 2 min in running water. Slides were stained in 1% pre-filtered Eosin for 5 minutes, dipped in ddH_2O and dehydrated in ethanol. Following histoclear immersion, slides were mounted using DPX and stored at RT.

2.2.11 Immunostaining of sectioned/ whole mount samples

Frozen slides were thawed for 40 min (RT), post-fixed with 4% PFA for 10 minutes and washed in 1XPBS. Slides/ whole mount samples were blocked (0.1% Triton X100 (Sigma Aldrich, UK), 1% bovine serum albumin (Sigma Aldrich, UK), 0.15% glycine (Fisher Scientific, UK) in 1XPBS) for 1 hour and

incubated in primary antibody (Table 2.1) diluted in blocking solution overnight at 4°C. Samples were washed in 1XPBS (RT). Secondary antibody (Table 2.2) was applied in blocking solution for 2 hours (RT) and washed in 1XPBS. Fluorescent immunolabelled slides were mounted using Vectashield (hard set with DAPI, Dako, UK). Slides were stored at 4°C.

Table 2.1: Chapter 2 primary antibodies.

Antibody	Species	Manufacturer	Concentration
TuJ1	Mouse	Covance	1:500
TuJ1	Rabbit	Covance	1:500
Hu C/D	Mouse	Invitrogen	1:500
HNK-1	Mouse	Hybridoma Bank (#3H5)	1:20

Table 2.2: Chapter 2 secondary antibodies.

Antibody	Species	Manufacturer	Concentration	Absorbance
Anti mouse	Goat	Alexa Fluor	1:500	568
Anti mouse	Goat	Alexa Fluor	1:500	488
Anti rabbit	Goat	Alexa Fluor	1:500	488
Anti mouse	Goat	Alexa Fluor	1:500	647
Anti rabbit/ HRP	Goat	Dako	1:250	N/A
DAPI	N/A	Sigma Aldrich	1:1000	350

2.2.12 Quantification of cell spread

In order to determine the extent of ENSC cell/ projection spread from the transplantation site within the developing neural tube, transplanted embryos were harvested at the timed intervals and sectioned as described above. Following staining with an anti-GFP antibody, GFP images of transplanted cells were collected using an ORCA-R2 cooled CCD camera mounted on an Olympus 1X70. The entirety of the spread along the anterior/ posterior axis was captured, and where appropriate composite images were collated into a tile scan using MosaicJ on FIJI. For each embryo, the section demonstrating the greatest spread was selected and measured along with the preceding and following section, and the average distance between the three calculated. For each age under examination, an n of three embryos was used.

2.2.13 DNA/RNA methods

2.2.13.1 RNA extraction

For tissue samples, the material was homogenized and immersed in Trizol (Invitrogen, UK). This was incubated with chloroform (RT) and centrifuged at 12,000x g for 10 minutes at 4°C. The upper aqueous phase was isolated, mixed with 70% ethanol and transferred to an RNeasy Mini spin column (Qiagen, UK). The manufacturer's protocol was then followed. For cells, the manufacturer's protocol was followed without modification. RNA yield was quantified using a Nanodrop.

2.2.13.2 cDNA synthesis

100ng of RNA was used for each reaction. This was added to 4µL 5X VILO reaction mix and 2µL 10X Superscript Enzyme Mix (Life Technologies, Paisley, UK). The volume was adjusted to 20µL of DEPC-treated water. Synthesis was conducted using a Thermofisher Cycler as per the manufacturer's protocol.

2.2.13.3 qRT-PCR

To determine cell composition and neurotransmitter competency of neurospheres, primers were designed with amplification product sizes of 100-200bp (Sigma Aldrich) (table 2.3). To test primer specificity, PCR reactions (table 2.4) using sample chick DNA were run and the products examined by gel electrophoresis (1% agarose dissolved in tris-acetate-EDTA (TAE) buffer with 0.005% ethidium bromide for visualization). Once predicted product sizes were confirmed the primers were used for qRT-PCR.

Table 2.3: Primers used for qRT-PCR

Probe target	**Primer sequence**	**Product size**	**Tm**
TuJ1	F: GCCCCGACAACTTCATTTT R: GCAGTCGCAGTTCTCACACT	138	63.8 63.4
p75	F: AGGTGATGGTGAAGGAGTGC R: GACGGTGGTGACAATGTCTG	183	64.2 64.3
S100	F: AGTACTCCGGGAAGGAAGGA R: GTCCAGTGCCTCCATGACTT	144	63.8 64.2
Sox10	F: AGCCTTCACAGGGTTTGCT	135	63.8

	R: GAGAGGCAGTGGTGGTCTTC		63.9
ChAT	F: AATGCCAGAACCAGAGCACA R: TCAGTCGTCAGCAAGCCAAT	189	65.8 66.0
GAD	F: GACATCCACCGCTAACACCA R: CGCCATCTTTATTCGACCATCC	131	66.0 68.3
GLS1	F: CTTACTCAAGCTTTCAGGAGGAA R: TGCCCATCCACTGTGCAAA	194	62.6 69.0
nNOS	F: ATGCTCAACTACCGCCTCAC R: AATGGCCCTCTTCTTGGTGG	117	64.2 67.3
TPH1	F: GTGCTGATGTACGGGTCTGA R: AGTTCATAGCCAGGTCTGCA	112	69.3 62.5
TPH2	F: CTCTATCCCACCCACGCTTG R: AACCGGTCTCACTGTGAAGC	159	66.7 64.3

qRT-PCR was used to verify primer accuracy and annealing conditions (table 2.5). Samples were assayed in triplicate and normalized to the house-keeping gene GAPDH, and analysed using the ABI Prism 7500 sequence detection system (Applied Biosystems) using Quantitect SYBR green PCR kit (Qiagen, Germany) according to the manufacturer's instructions.

Table 2.4: Chapter 2 PCR cycling programme

Step	Temp °C	Time
1	94	3 minutes
2	94	30 seconds
3	60	1 minute
4; go to step 2, 35 cycles	72	30 seconds
5	72	2 minutes
6	4	Hold

Table 2.5: Chapter 2 qRT-PCR cycling programme

Step	Temp °C	Time
1	95	15 min
2	95	20 seconds
3	60	30 seconds
4; go to step 2, 40 cycles	72	30 seconds

2.2.14 Statistical analysis

qRT-PCR data was analysed by Student's t test (two-tailed). Cell spread data was analysed using both Student's t test (two-tailed) and analysis of variance (ANOVA) Survival of transplanted and non-transplanted groups was compared using the Log-rank (Mantel-Cox) test. GraphPad Prism software was used for all analyses. p values of <0.05 were taken as significant.

2.3 Results

2.3.1 Vagal GFP neural tube transplantation effectively and stably labels neural crest cells and their derivatives

GFP/ WT chimeras were generated at E1.5 to label vagal NCCs and their derivatives with GFP, allowing FACS isolation of neural crest derived ENSCs. Following grafting of GFP neural tube into WT chick embryos, chimeras were returned to the incubator to develop to the appropriate stage. 1 day after grafting, streams of GFP+ cells could be seen migrating to the future foregut (Fig.2.4). An additional concentration of GFP+ cells was observed in the region of the circumpharyngeal crest, presumably on route to the developing heart.

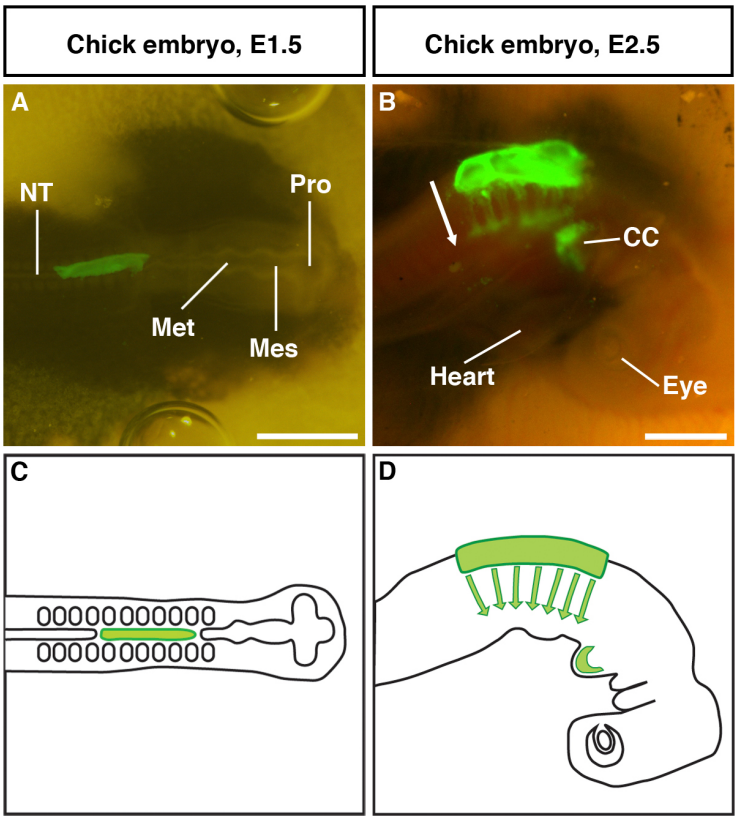

Figure 2.4: Fluorescence microscopy visualisation of early migrating enteric NCCs following grafting at E1.5.

GFP/WT chimeric grafting allows the visualization of NCCs in ovo. Following vagal grafting at E1.5 (A), GFP+ NCCs leave the neural tube and migrate in streams towards the developing gut (B). GFP+ cells can also be seen circumpharyngeal crest (CC), on route to the developing heart. C, D, cartoons of grafted chick embryos. NT - neural tube, Met – metencephalon, Mes – mesencephalon, Pro – prosencephalon. Scale bars, 1mm.

Chimeras were initially sacrificed at E6.5 to ensure cells had been correctly labeled, that they were developing/migrating as expected, and were forming structures comparable to that of wild type equivalents (Fig. 2.5). The grafting procedure labeled neural crest-derived structures, allowing clear distinction from surrounding tissue without neural crest contributions (Fig. 2.5, A). At E6.5 GFP+ ENCCs can be seen migrating through the developing gut (Fig. 2.5, B). The migration wavefront has just passed the caecal buds (Fig. 2.5, first inset, arrow). Distal to the wavefront, GFP+ cells are immunopositive for the marker TuJ1 (Fig. 2.5, second inset, arrowhead), indicating differentiation towards a neuronal lineage.

To further confirm GFP+ labeling of neural crest-derived cells, cross-sections of chimeric guts were analysed for presence of GFP+ structures. Chimeras were sacrificed at E14, gelatin embedded and cryosectioned to visualise the enteric plexuses. GFP+ cells co-label for the neuronal marker TuJ1 (Fig. 2.5, C) and the neural crest marker HNK-1 (Fig. 2.5, D) within the colon of chimeric chick embryos (proximal and distal small intestine showed similar staining, data not shown). At E14, the entire GI tract was colonized with GFP+ enteric NCCs, with the ENS clearly labeled with GFP throughout (Fig.2.6).

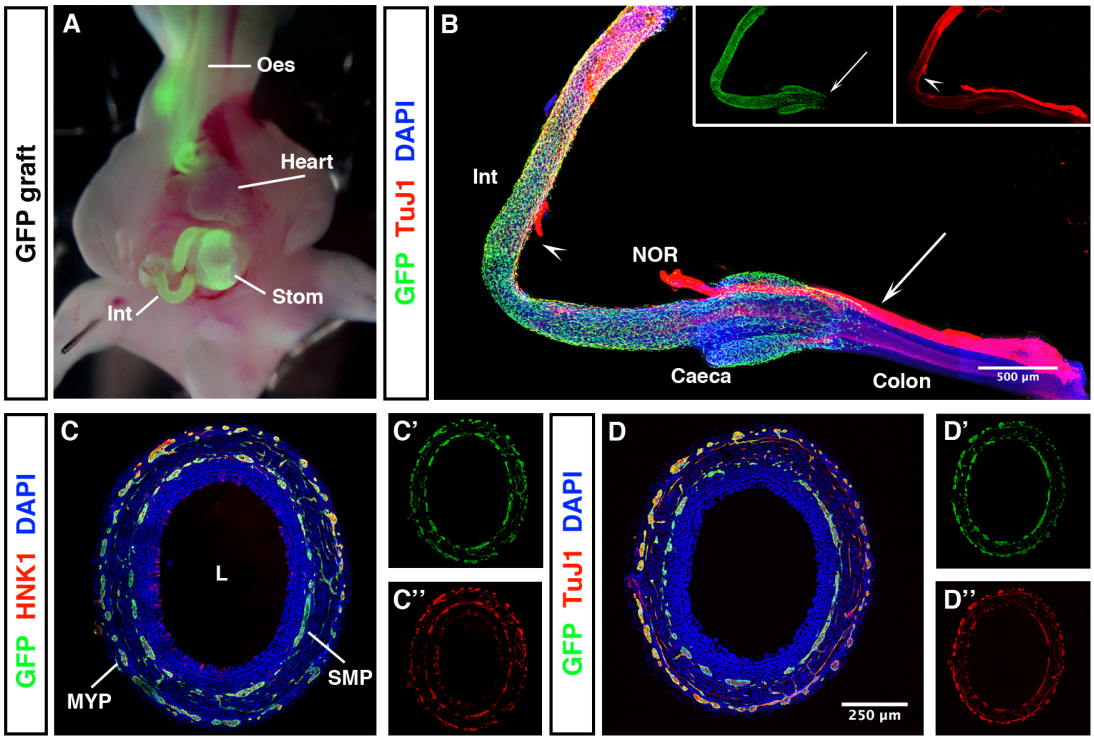

Figure 2.5: Immunofluorescent detection of GFP+ enteric NCCs following chimeric grafting.

Vagal grafting results in specific labeling of neural crest-derived tissues such as the ENS of the GI tract, providing clear distinction of surrounding tissue (A). At E6.5, GFP+ cells have entered the gut and migrated beyond the caeca (B, arrow and first inset). Distal to the migration wavefront, TuJ1+ neurons were identified (B, arrowhead and second inset), indicating differentiation towards a neuronal lineage. A portion of the nerve of Remak (NOR) can be seen adjacent to the colon. By E14.5, the GI tract appears completely colonized by migrating neural crest cells. Transverse colon sections reveal the formation of GFP+ myenteric and submucosal plexuses (C, D). GFP+ cells co-express the neuronal marker TuJ1 (C'') and the neural crest cell marker HNK-1 (D'').

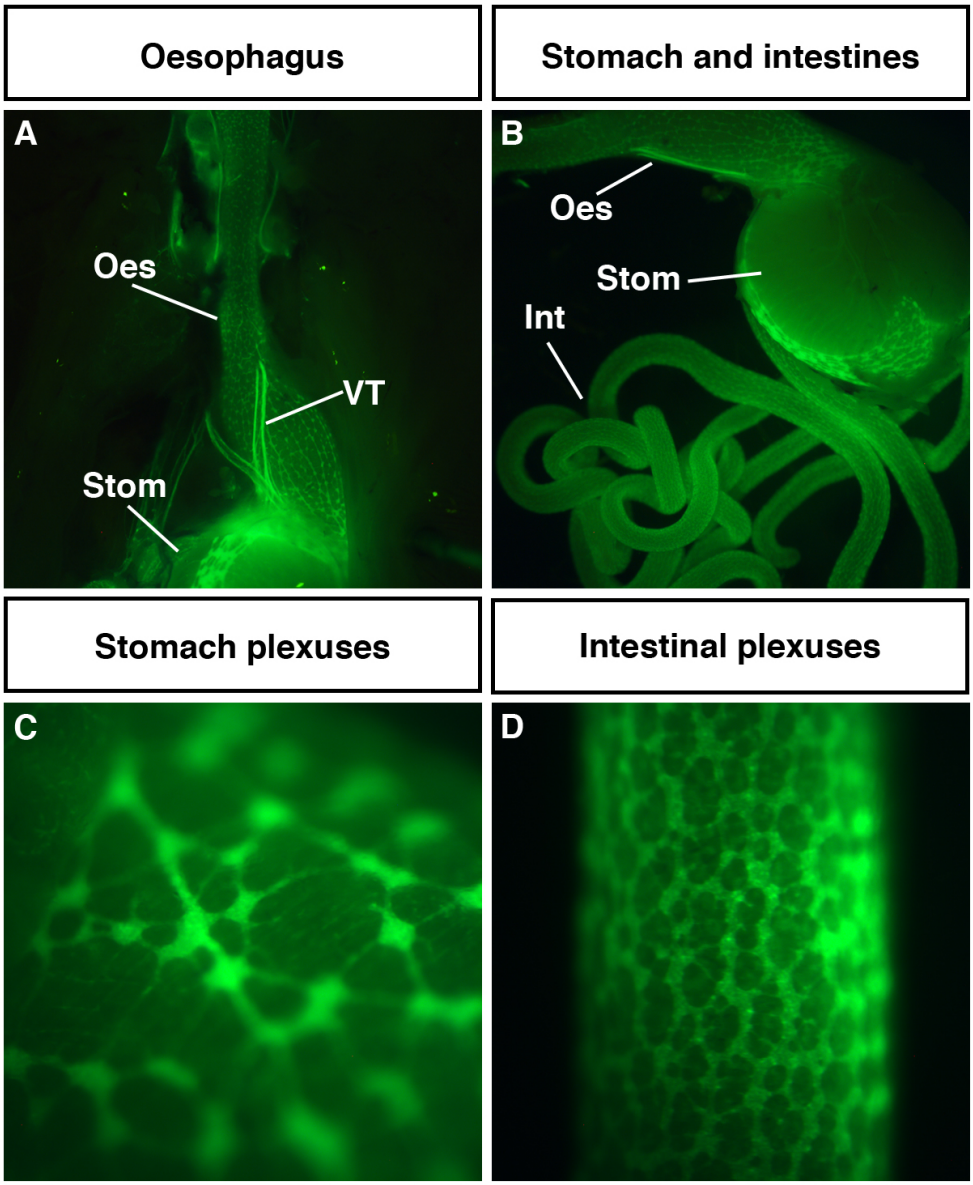

Figure 2.6: Macroscopic examination of GFP+ enteric NCC colonization of the GI tract following vagal grafting at E1.5.

Following grafting of a GFP+ vagal neural tube into a WT chicken with the corresponding region ablated, GFP+ NCCs migrated into and along the gut to form the ENS. Starting orally, NCCs colonise first the oesophagus (A), and, moving aborally, the stomach and intestines (B). GFP labelling of the ENS was robust and strong, enabling ready identification of plexuses in the stomach (C) and intestines (D).

2.3.2 Isolation and characterization of GFP-labeled ENS precursors and stem cells

FACS was used to isolate GFP+ ENCCs (including ENSCs and their derivatives) from surrounding gut tissues at E14, (Fig. 2.7, A). ENCCs could be clearly identified by their strong GFP+ signal. To eliminate dead (and therefore potentially autofluorescent cells) the positive collected population was further restricted by size. Isolated GFP+ cells were cultured to form neurospheres, allowing ease of transplantation into the spinal cord and subsequent detection. Within 2-3 days of initial *in vitro* culture GFP+ cells formed interconnected aggregates, including extension and connection of axonal processes. These aggregates typically formed neurospheres after 1 week in culture, which become free-floating ~1 week later. These neurospheres stained positive both for the neural crest marker HNK-1 (data not shown) and for the neuronal marker TuJ1, confirming that they were of neural crest origin, and that differentiated cells could be found within the spheres (Fig. 2.7).

To assess the potential for *in vivo* integration of transplanted enteric-derived neurospheres (henceforth referred to as ENSC-derived neurospheres) with endogenous CNS cells in the injured spinal cord, cultured GFP+ ENCCs and CNS-derived cells were co-cultured *in vitro*. CNS-derived cells were labeled with an mCherry lentiviral vector (labeling efficiency 71.2±6.8%), and the two populations cultured together for 1 week. CNS cells and enteric cells integrated extensively (Fig. 2.7, C), including the extension of ENS/ CNS axons alongside and towards one another (arrows). Mixed-population neurospheres were also observed (Fig. 2.7, D).

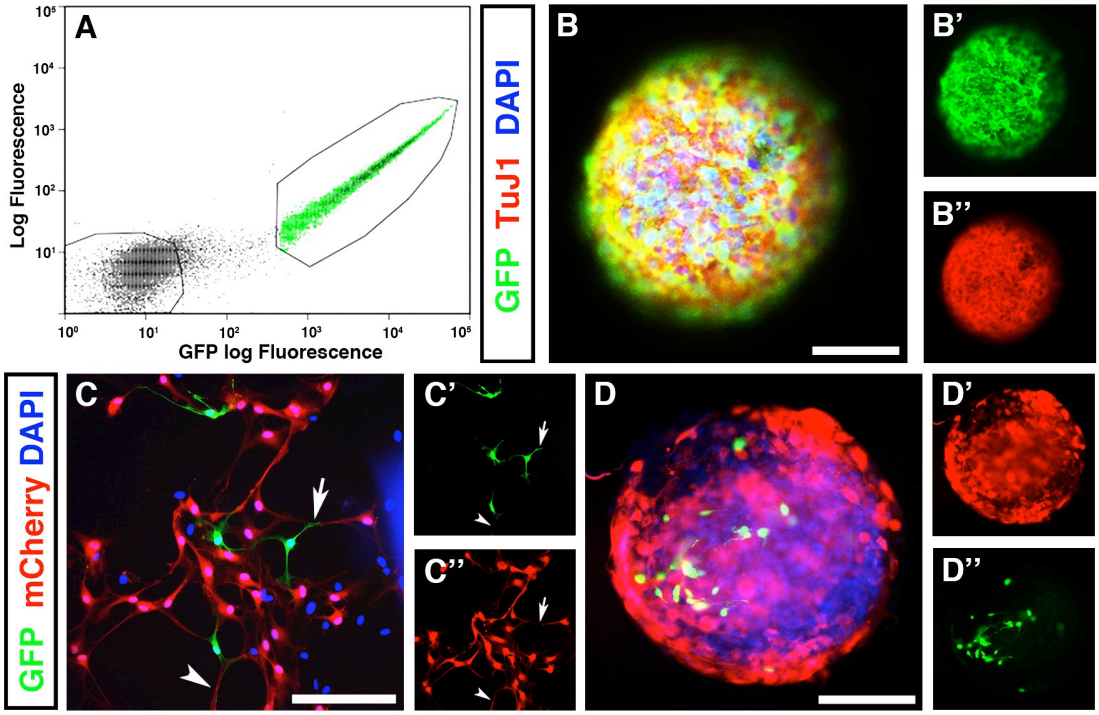

Figure 2.7: *In vitro* analysis of ENSCs and their compatibility with SC-derived cells.

After grafting, embryos were allowed to develop to E14 before harvesting. Intestines were dissociated into a single cell suspension and sorted based on GFP expression and size (A). Negative populations were collected and cultured as controls. Following FACS isolation, GFP+ graft-derived cells formed free-floating neurospheres after 1-2 weeks in culture. Cells within the neurospheres were mostly immunopositive for the neuronal marker TuJ1 (B). Graft-derived GFP+ ENSCs integrated with spinal cord-derived cells (labelled with an mCherry lentiviral construct), including formation of close associations and extension of cell projections and axons (C). C′ and C′′ show higher magnification selections of C, and highlight CNS and ENS axons extending alongside and towards one another. After several days in culture ENS/CNS cells aggregated in clusters to form mixed-population neurospheres (D). Scale bar: B, C - 50μm, D - 100μm.

The close association of processes observed in these co-culture experiments suggested the possibility of ENSC- and SC-derived cell communication, and previous studies have demonstrated the ability of the ENS to produce many of the same neurotransmitters as those utilised in the CNS. However, the relative expressions of neurotransmitters in central and enteric-nervous system-derived cultures have not, to our knowledge, been directly compared. Uncultured gut and spinal cord tissue was therefore analysed by qRT-PCR to determine cell composition. Independently cultured ENSC neurospheres were then also analysed and compared to uncultured preparations of spinal cord tissue.

Gene expression analysis of the major cell types typically found within neurospheres revealed common expression of TuJ1 (neurons), S100 (glia), Sox10 (stem cells), and p75 (neural crest cells), in both gut and SC-derived tissue. Levels of S100 and Sox10 expression were significantly higher in SC-derived tissue compared to gut tissue (0.055 *vs* 0.040, p=0.0098, and 0.100 *vs* 0.045, p=0.0049, respectively), while TuJ1 expression was significantly higher (0.230 *vs* 0.059, p=0.0006) in SC-derived tissue (Fig. 2.8, A). Similarly, in comparisons of SC and cultured ENSC expression levels, relative expression of TuJ1 was dramatically higher in SC samples than in ENSC-derived neurosphere populations (0.236 *vs* 0.058, p=0.049), which appeared similar to expression levels of uncultured gut tissue (Fig. 2.8, B). Levels of S100 expression in ENSC-derived neurospheres also appeared similar to levels found within the gut (0.033 *vs* 0.040, p=0.07). However, compared to relative expression of Sox10 in uncultured gut, levels within enteric neurospheres increased to higher levels than that seen in SC tissue, although this was not significant (0.045 *vs* 0.225, p=0.0753). These results for enteric neurospheres are comparable to published data collected by our lab and others. Binder *et al* and Cooper *et al* both used immunostaining to reveal

similar proportions of neurons and glia in a p75+ FAC-sorted population (Binder et al., 2015; Cooper et al., 2016). Metzger *et al* also revealed comparable levels of TuJ1+ neurons and S100+ glia, although at lower levels (possibly due to differences in generation of cell cultures; manual dissociation of myenteric plexus-containing muscle strips was used in place of the FACS-isolation utilized in the previous studies) (Metzger et al., 2009a).

To determine which neuronal subtypes were contained within the TuJ1+ population, relative expression levels of 5 neurotransmitters, considered of key importance for spinal cord injury based on published literature, were examined. nNOS (NO), TPH1 (serotonin), GLS1 (glutamine), ChAT (acetylcholine) and GAD (GABA) were expressed in both gut and SC tissue. Relative expression of nNOS, TPH1, GLS1 and ChAT was comparable between gut and SC-derived tissue, with significantly higher expression of GAD (GABA) in SC tissue (Fig. 2.8, C). Acetylcholine expression was highest in both populations. Relative expression of all neurotransmitter markers stayed broadly similar in cultured ENSC-derived neurospheres as for uncultured gut tissue, with only a non-significant decrease in nNOS (0.041 *vs* 0.059, p=0.051), and a slight, but significant decrease in serotonin expression (0.025 *vs* 0.033, p=0.049) in ENSC cultures (Fig. 2.8, D).

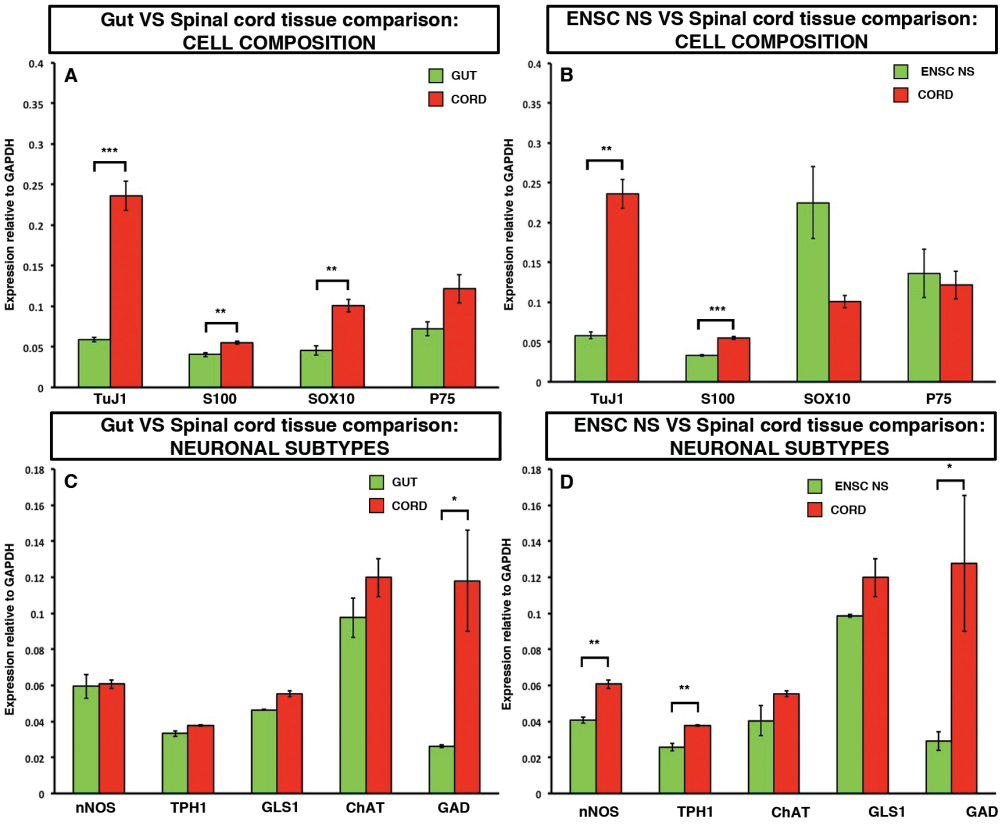

Figure 2.8: qRT-PCR analysis of cultured ENSC and whole gut/spinal cord samples.

The glial marker S100, stem cell marker Sox10 and neural crest marker p75 were expressed at similar levels in both samples, with significantly higher levels of the neuronal marker TuJ1 in the SC (A). Expression levels of cultured ENSC-derived neurospheres were comparable to gut samples, albeit with increased expression of SOX10 in ENSC cultures (B). Expression levels of specific neuronal subtypes were also analysed by qRT-PCR, revealing common expression between uncultured gut and SC samples of nNOS (NO), TPH1 (serotonin), GLS1 (glutamine) and ChAT (acetylcholine) and GAD (GABA), with significantly higher levels of GAD in the SC (C). Expression levels of the same neuronal subtype markers were broadly the same for ENSC-derived neurosphere cultures as for uncultured gut samples, with only a non-significant decrease in nNOS, and a slight, but significant decrease in serotonin expression in ENSC cultures (D). nNOS, TPH1, GLS1, GAD n=3, ChAT n=2.

2.3.3 GFP+ ENSC neurosphere transplantation into early neural tubes

The immature chick neural tube environment, upon injury, is far less hostile than that of the adult. A lack of an immune response (Negash et al., 2004; Seto, 1981) or presence of myelin (Macklin WB and CL, 1985), combined with scarcity of blood vessels in the chick at E1.5 (Bellairs and Osmond, 1998), make the chicken embryo an idea model in which to test the ability of ENSCs to survive in an immature spinal cord environment. At E1.5, a small segment of the neural tube (one somite length, at somite 7) was ablated, and an equivalent-sized neurosphere grafted into the 'injured' region (Fig. 2.9). Following transplant, chicks were harvested at E5.5, 7.5 and 9.5 to assess the degree of cell spread, integration and differentiation. At each time point, embryos were harvested for both longitudinal and coronal sectioning and stained for the neuronal marker TuJ1 (for longitudinal sectioning at each time point, n=3). There was no difference in survival between transplanted and non-transplanted groups (log-rank Mantel-Cox test, $p=0.5607$, n=13 transplanted embryos, n=5 injured without transplant).

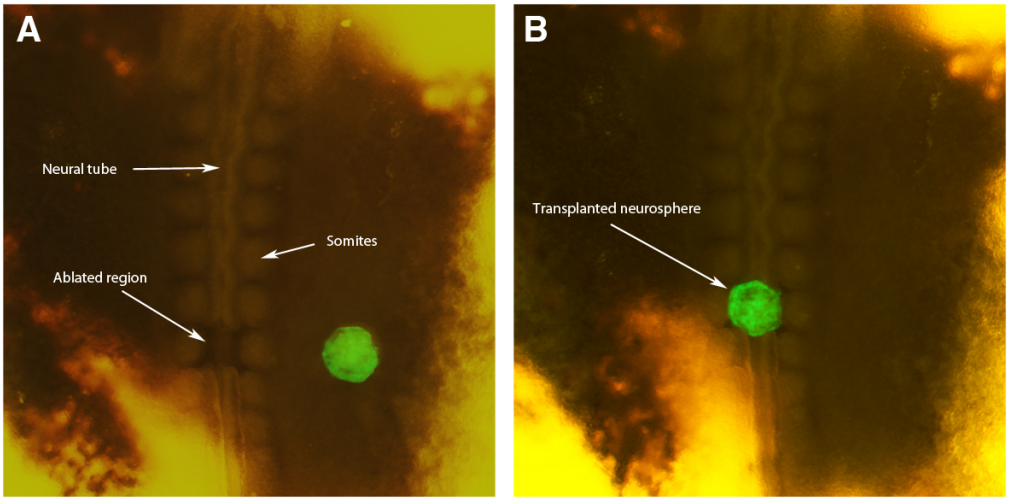

Figure 2.9: GFP+ neurosphere grafting into the developing neural tube of early stage chick embryos.

At E1.5, a region of the neural tube equivalent to the length of 1 somite was microsurgically removed at the level of somite 7 (A) and a GFP+ neurosphere transplanted into the void (B). Embryos were then allowed to develop to the desired stage and harvested for further analysis.

Upon examination under a stereomicroscope at E5.5, GFP+ cells had spread through the tissue (Fig. 2.10, A, B). Cells were localized to the cervical region of the spinal cord, as expected following vagal transplantation at somite 7. The strongest GFP signal was deep within the tissue. Coronal sectioning revealed numerous GFP+ transplanted cells within the spinal cord (demarcated by TuJ1 staining, red, Fig. 2.10, D). GFP+ cells surrounded the entirety of the cord with roughly equal distribution dorsally and ventrally. The vast majority of transplanted cells were localized to the white matter, with only occasional cells found within the grey matter. Cells were also found within the dorsal root ganglia and other spinal cord projections. Rarely, a small number of cells were found in the mesenchymal tissue between the spinal cord and dorsal dermis. Longitudinal sections of transplants harvested at E5.5 revealed transplanted cells mostly localized to the injury zone,

extending, and potentially forming bridging connections between, the anterior and posterior spinal cord tissue. Again, spread into the surrounding dorsal root ganglia was observed.

Embryos harvested at E7.5 revealed a much greater spread of GFP+ cells from the transplantation site (Fig. 2.11, A, B). Coronal sections showed similar white matter localization to embryos harvested at E5.5 (Fig. 2.11, C). However, at E7.5 transplanted cells were predominantly found towards the dorsal aspect of the spinal cord. This finding was replicated in longitudinal sections, in which the vast majority of transplanted cells were found in the dorsal-most sections. Again, these were found forming bridging structures between the anterior and posterior spinal cord tissue, in the region of the injury zone (Fig. 2.11, D). Analysis of embryos harvested at E9.5 revealed an almost exclusive localization of transplanted GFP+ cells to the dorsal presumptive white matter, continuing the trend observed between E5.5 and E7.5 (Fig. 2.12, A). Within dorsal longitudinal sections, GFP+ cells were still observed to form continuous structures between the anterior and posterior SC tissue (Fig. 2.12, B).

At E13.5, GFP+ cells had spread substantially along both the anterior and posterior spinal cord tissue (Fig. 2.13, A). Transverse sections throughout the spinal cord at E13.5 reveal that, at the injury zone, transplanted cells showed no grey/white matter localization preference (Fig. 2.13, C). However, moving anteriorly GFP+ cells became progressively localized to the white matter only (Fig. 2.13, B). The presence of robust bridging structures was noted in several embryos (Fig. 2.13, D, arrow).

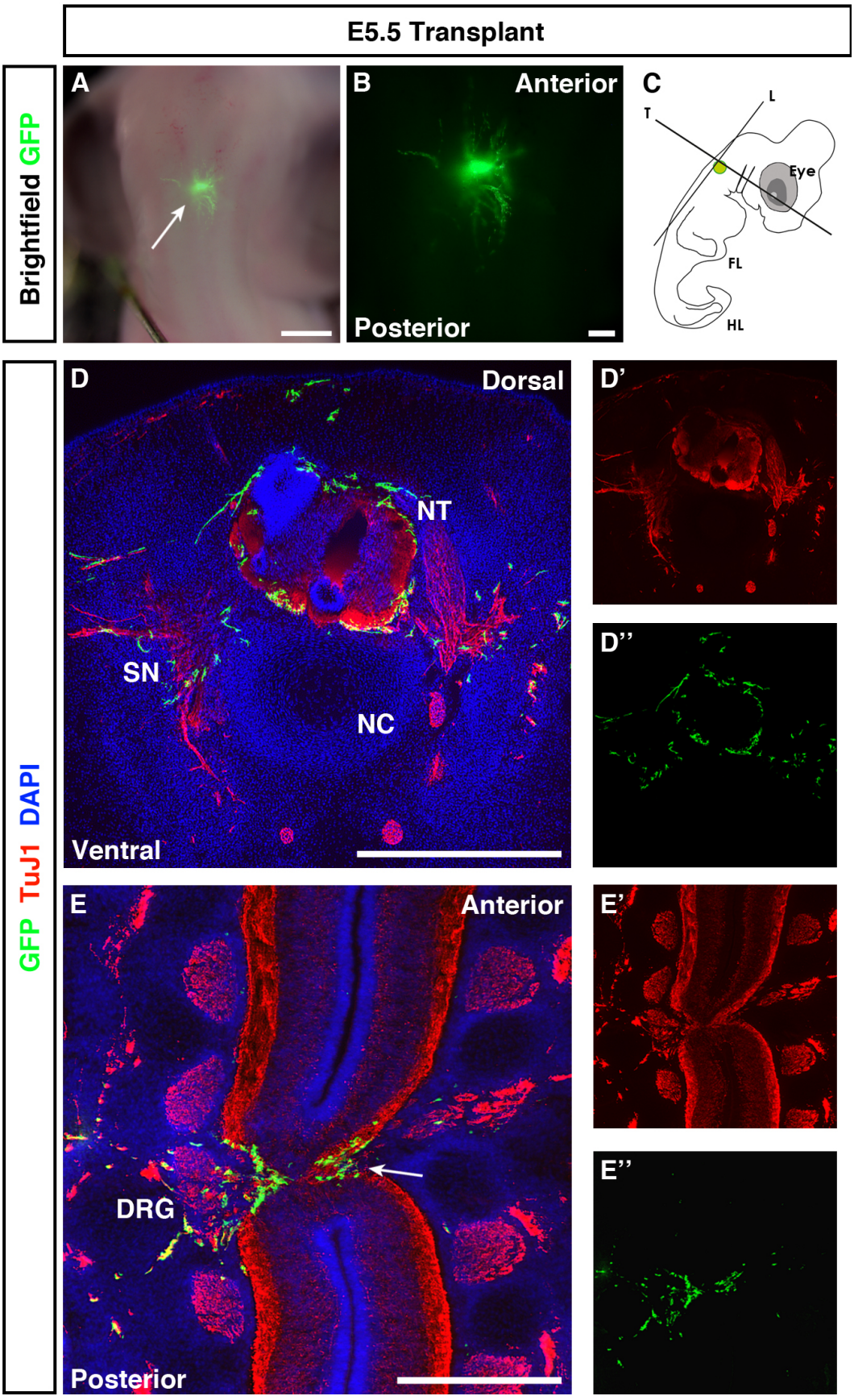
E5.5 Transplant
Brightfield GFP
A
B
Anterior
Posterior
C
L
T
Eye
FL
HL
GFP TuJ1 DAPI
D
Dorsal
NT
SN
NC
Ventral
D'
D''
E
Anterior
DRG
Posterior
E'
E''

Figure 2.10: Immunofluorescent analysis of E5.5 cryosectioned embryos transplanted with ENS-derived neurospheres.

Stereoscopic examination revealed GFP+ cells spreading from the transplantation site (A, B). C shows a schematic of transplantation site and sectioning planes for analysis. Co-staining of coronal sections with GFP and TuJ1 revealed localisation of transplanted cells to neuronal rich regions. 4 days after transplanting, cells had formed a 'halo' around the spinal cord, staying almost exclusively in the presumptive white matter (D). Longitudinal sections revealed transplanted cells had formed bridging connections through the injury zone, between the anterior and posterior spinal cord tissue (E, arrow). In both coronal and longitudinal sections, GFP+ cells spread into the PNS through the dorsal root ganglia (DRG, D, E). Insets show high power magnification revealing numerous GFP+ extensions from the transplanted neurosphere. FL – forelimb, HL – hindlimb, NT – neural tube, SN – spinal nerve, NC – notochord, DRG – dorsal root ganglia. Scale bar: A - 3mm, B – 1mm, D, E - 500μm.

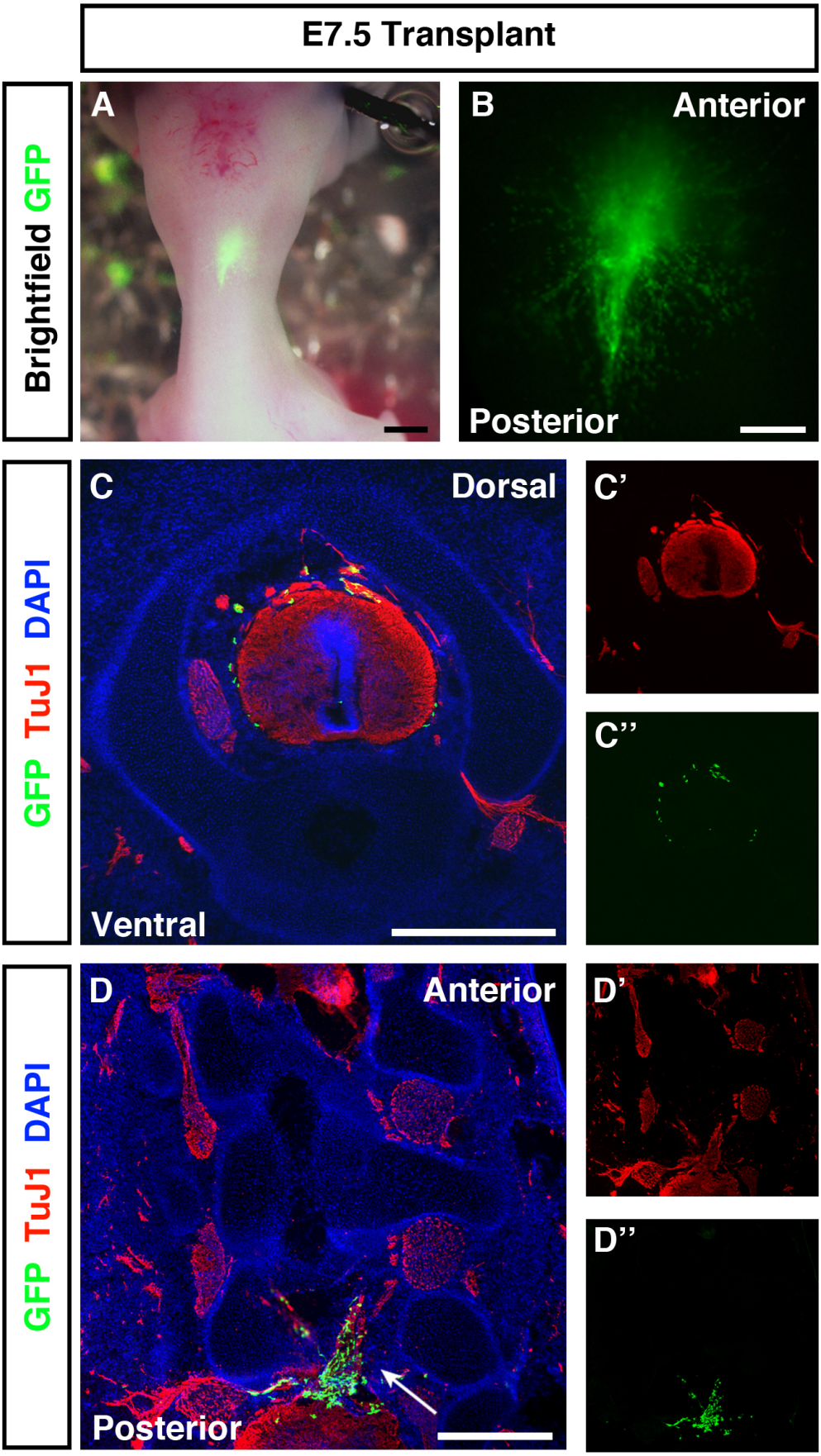
E7.5 Transplant
Brightfield GFP
A
B
Anterior
Posterior
GFP TuJ1 DAPI
C
Dorsal
Ventral
C'
C''
GFP TuJ1 DAPI
D
Anterior
Posterior
D'
D''

Figure 2.11: Immunofluorescent analysis of E7.5 cryosectioned embryos transplanted with ENS-derived neurospheres.

Fluorescent stereoscopic examination of embryos harvested at E7.5, 6 days after transplant, reveals extensive GFP+ cell spread from the transplantation site (A, B). Coronal sections show the presence of GFP+ cells in a 'halo' within the future spinal cord white matter, in a pattern similar to that seen at E5.5 (C). However, at E7.5 transplanted cells appear to show a preferential distribution around the dorsal spinal cord, with very few cells at the ventral side. Longitudinal sections reveal GFP+ cells forming bridging connections between the anterior and posterior segments of the injured spinal cord (square bracket, D). In the longitudinal section, a few of GFP+ cells spread into the dorsal root ganglia (DRG). Scale bar: A, B – 1mm, C, D - 500μm.

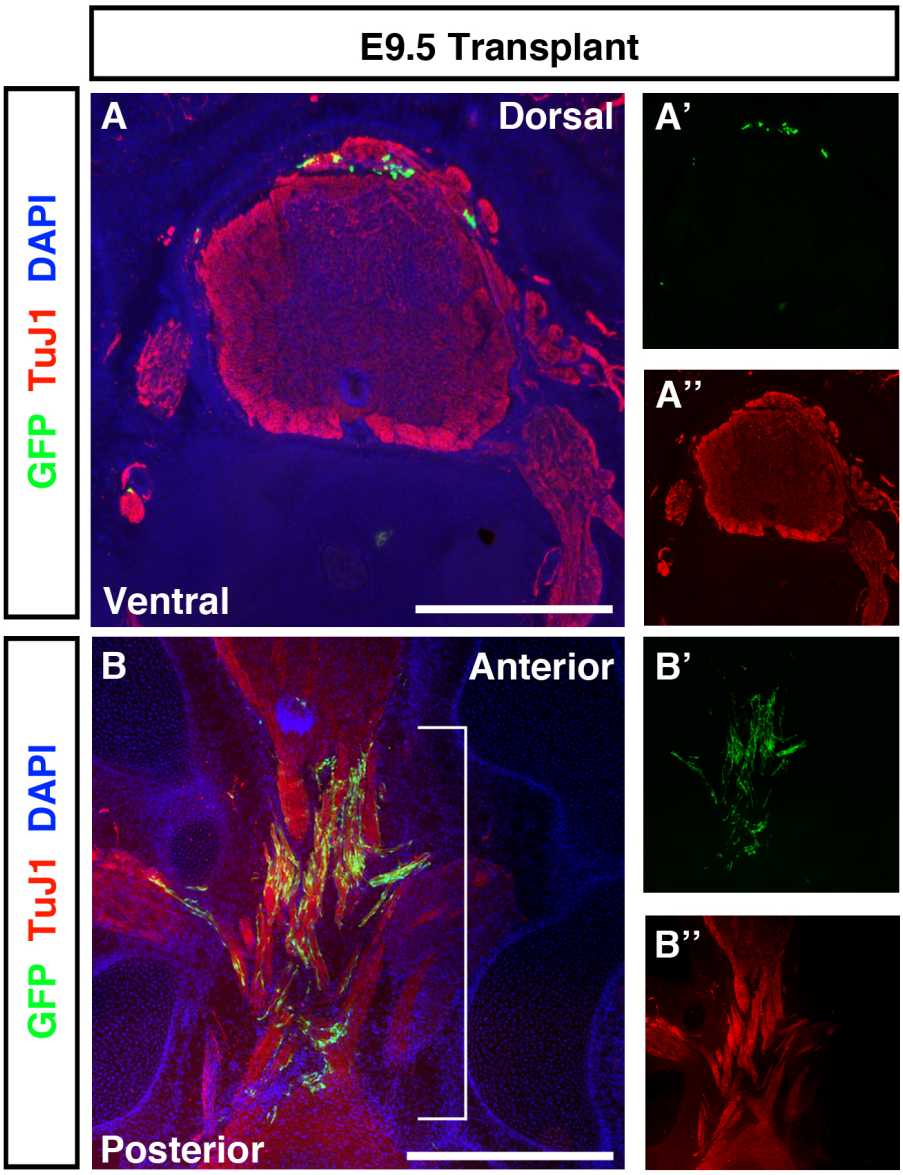

Figure 2.12: Immunofluorescent analysis of E9.5 embryos transplanted with ENS-derived neurospheres.

Coronal sections of embryos harvested 8 days post-transplant at E9.5 show GFP+ cells localised almost exclusively to the dorsal spinal cord, with only rare cells found more ventrally (A). Longitudinal sections reveal GFP+ cells forming bridging connections between anterior and posterior spinal cord tissue (square bracket), consistent with immunofluorescent examination of transplants harvested at E5.5 and E7.5 (B). The majority of cells appear localised to the white matter. Scale bars: A, B - 500μm.

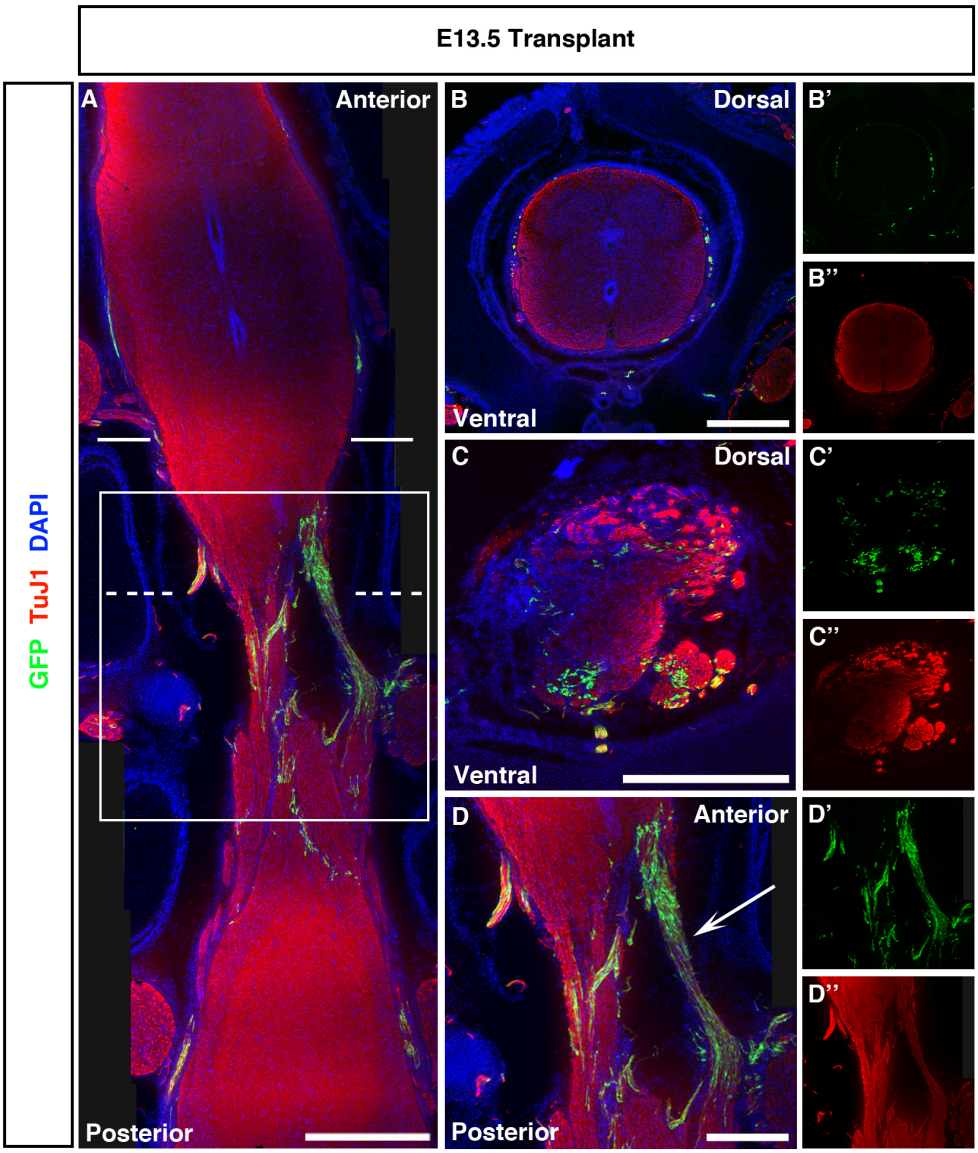

Figure 2.13: Immunofluorescent analysis of GFP+ bridging structures across the injury site of E13.5 embryos transplanted with ENS-derived neurospheres.

The extent of transplanted GFP+ cell spread is shown along the anterior posterior axis (tiled images, A). The solid and dashed lines in A show the approximate levels of the transverse sections shown in B (anterior to the injury zone) and C (through the injury zone). The solid box in A indicates a higher magnification of the injury zone and bridging structure shown in D. Scale bars: A – 1mm, B, C, D - 500μm.

Many non-NSC have been proposed and indeed tested for their potential to aid in repair of the injured spinal cord. While these have shown benefit, an advantage of a NSC source such as ENSCs is their predicted ability to produce appropriate cell types, and for these to function as either relay circuit components or bridging structures. In order to determine the fate of cells post-transplant, GFP+ cells within the injury zone were examined at higher magnification (Fig. 2.14). In embryos examined at E5.5, transplanted cells frequently co-labelled for the pan-neuronal cell marker TuJ1 and GFP, demonstrating differentiation towards a neuronal lineage (Fig. 2.14, A). Further, transplanted cells were not found to differentiate towards a glial lineage, and were not found in areas of GFAP+ staining (Fig. 2.14, B).

For transplanted neurons to function efficiently as either circuits or bridges, alignment along the anterior/ posterior axis is almost certainly advantageous. The majority of spinal tracts run from the brain and along the anterior/ posterior axis of the spinal cord to the body, or vice versa, with only a few tracts (such as spinal reflex circuits) arranged perpendicular to the anterior/ posterior axis. Therefore, these longitudinal tracts are by far the most likely to be damaged in SCI, and the most in need of repair. Analysis of transplanted ENSCs within the injury zone revealed numerous cell bodies aligned along the anterior posterior axis, often but not always within residual TuJ1+ neuronal tracts (Fig. 2.14, C). Quantification of maximal longitudinal spread across embryos harvested at all time points revealed a progressive increase in spread with time post-transplant, with the greatest anterior-posterior spread within transplants harvested at E13.5. Compared to an average spread of around 996.3μm at E5.5, embryos harvested at E13.5 showed an average spread of 7373.2.3μm (n=3). The increase in spread from E5.5 was significant compared to both E9.5 ($p=0.0077$) and E13.5 ($p=0.0127$). ANOVA comparison across all groups was also significant

(p=0.002) (Fig. 2.9, D).

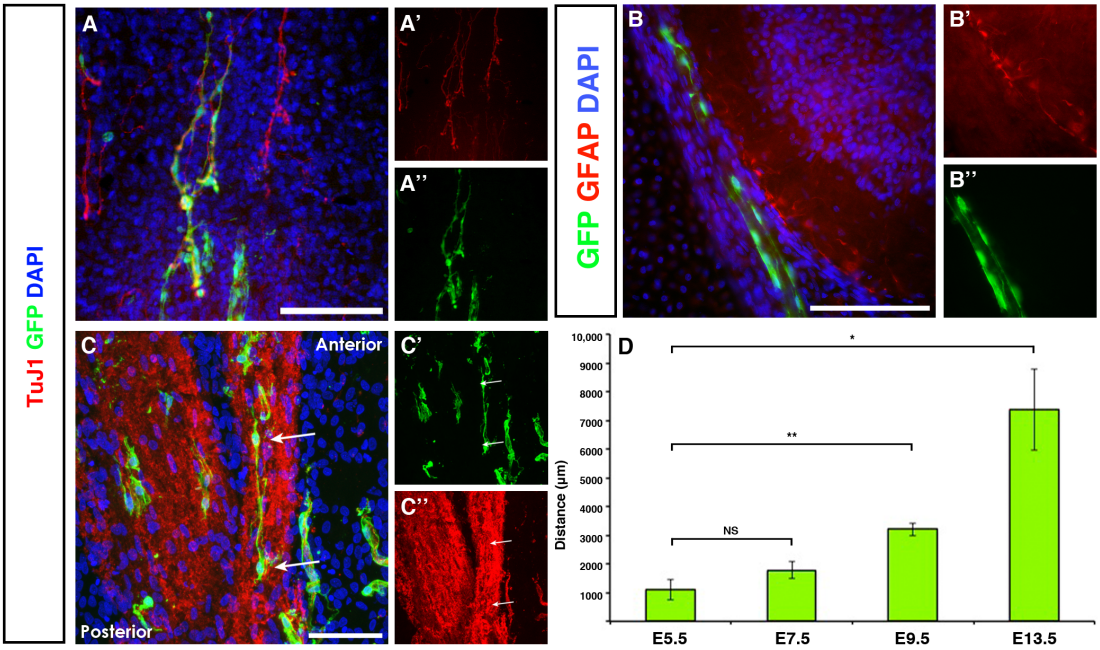

Figure 2.14: Cell fate analysis of transplanted ENSCs.

Longitudinal cryosections of chick embryos harvested 12 days after transplantation at E1.5 with a GFP+ ENS-derived neurosphere reveal TuJ1+/GFP+ cells projecting through the spinal cord injury zone (A). In contrast, transplanted cells were distinct from areas of GFAP staining, with no GFAP+/GFP+ cells observed (B). Particularly in transplanted embryos harvested at later stages, longitudinal sections showed GFP+ cells within the injury zone, often adhered to residual TuJ1+ structures between the anterior and posterior spinal cord tissue (although cells were also found within TuJ1- injury zone tissue). These usually aligned along the anterior/ posterior axis of the spinal cord (C). Quantification of GFP+ spread along the anterior-posterior axis across the four time points under examination reveals a progressive increase in spread with increasing time post-transplant (C). Compared to an average spread of approximately 996.3µm at E5.5, embryos harvested at both E9.5 and E13.5 showed significantly higher cell spread. * =<0.05, **=<0.005.

2.3.4 GFP+ ENSC neurospheres transplanted into later stage (E11) neural tubes survive and extend axons into the white matter after only 24 hours

To allow manipulation of chick embryos at a later stage of development, chicks were cultured *ex ovo* until E11, at which point a severe cervical injury was induced. GFP+ neurospheres were transplanted into the injury site and the embryos allowed to develop for 24 hours before harvesting. Higher mortality rates were observed for late compared to early stage transplants. In three of four surviving transplants, the transplanted neurosphere was found adjacent to the spinal cord. No GFP signal was detected in the fourth. Figure 2.15 shows such localization to the white matter of the cord, including the extension of processes (arrow) into the presumptive white matter even after only 24 hours.

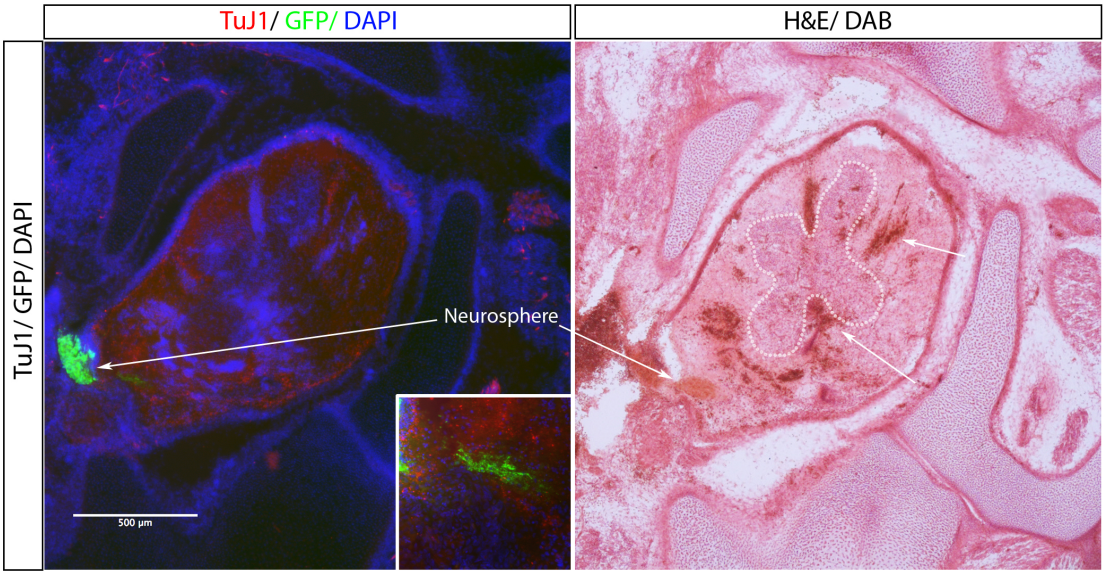

Figure 2.15: Immunofluorescent/histological examination of embryos transplanted at E11 with a GFP+ ENS-derived neurosphere.

Coronal cryosections of a chick embryo injured and transplanted with a GFP+ neurosphere at E11 and harvested 24 hours later show the neurosphere localized to the cord (arrow). In the above example, GFP+ processes extend into the white matter. H&E/ DAB staining of an adjacent section confirms the presumptive white matter localization of transplanted cells (grey matter indicated by dashed line). Blood deposits in the cord gave strong background staining (unlabeled arrows), and also seemed mostly restricted to the white matter).

2.4 Discussion

2.4.1 Summary

The results presented provide preliminary evidence that stem cells, derived from the ENS of the GI tract, can survive and integrate in the embryonic spinal cord, encouraging further assessment of their potential as a stem cell source for SCI therapy.

The chick model allows access to the developing embryo, permitting generation of GFP/ WT chimeras (Delalande et al., 2015). This method was utilized successfully to label vagal neural crest derivatives, including the ENS and its stem cell population. GFP+ cells and their derivatives were isolated using FACS and expanded in culture to form GFP+ neurospheres. ENS cells, including ENSCs integrated with CNS-derived cells *in vitro*, implying the potential for functional integration *in vivo*, and qRT-PCR analysis revealed common expression of neurons, glia, stem cells and a subset of neuronal subtypes between spinal cord tissue and gut/cultured ENSC samples. Finally, GFP+ neurospheres transplanted into the neural tube migrated through the spinal cord white matter and injury site, potentially forming bridging connections. This spread increased along the anterior/ posterior axis with increasing time post transplantation, and included extension of fine processes.

2.4.2 Efficient isolation of ENSCs and subsequent co-culture with SC-derived cells

Previous work has shown the specificity of GFP/WT neural tube grafting labelling of ENCCs and their derivatives (Delalande et al., 2015). The data presented here is in line with previous publications demonstrating formation of the ENS from the vagal region of the neural crest, and lack of GFP+ ENCC migration to non-ENCC-derived structures (Burns et al., 2002; Burns and Le Douarin, 2001; Delalande et al., 2015). In addition, this chapter provides further evidence that the differentiation wavefront, as demarcated by TuJ1 staining, lags significantly behind the migration wavefront of ENCCs as they colonise the GI tract. This is in agreement with publications demonstrating both the tendency of TuJ1+ cells to be found distal to the migration wavefront, and to migrate more slowly than their undifferentiated counterparts (Hao et al., 2009; Young et al., 2002; Young et al., 2005). As revealed by cross sections of colons from chimeric embryos, the grafting procedure resulted in robust labelling of ENCCs and their derivatives, evidenced by co-localization with both the neural crest cell marker HNK1 and the neuronal marker TuJ1. This translated into efficient cell isolation by FACS. Typically between 3-5% of total 'events' were collected. Previously published data stated the percentage of neural crest-derived cells within the mouse gut to be around 7-8% of total gut cells at the age of chimeric chick embryo harvest within the study (E14) (Binder et al., 2015). In this study, a Wnt1-Cre;Rosa26$^{Yfp/Yfp}$ mouse line was used, labelling with near-100% efficiency all neural crest cells (Binder et al., 2015; Jiang et al., 2000). The total number of GFP+ cells within the gut of GFP/WT chimeric chick embryos in the current study was likely higher than the 3-5% collected following FACS, as the sort gating was set to a highly restricting level to ensure purity of the collected

population. The percentage of FACS+ cells collected in the current study therefore likely demonstrates a similarly high level of labelling efficiency to previous publications.

Co-culture experiments of isolated ENSCs and their derivatives with SC-derived cells demonstrated the propensity of the two populations to interact. This experiment neatly displays the potential of ENSCs to integrate with endogenous networks within the spinal cord following transplantation. Stem cell transplantations have induced functional improvements in SC-injured recipients, as assessed by BBB evaluation and electrophysiological recordings across the injury site following transplantation of umbilical mesenchymal stem cells (Lu et al., 2012) or stepping performance on the horizontal ladder test following neural stem cells (Hong et al., 2014), among others. A leading theory of the mechanism by which this occurs is through establishment of lesion-spanning stem cell-based bridges for endogenous axons to cross (Popovich, 2012). In this study, extension of ENSC axons alongside SC-derived cell axons was frequently observed, implying the ability of ENSCs to function as such bridges. This is also supported by the extension of axons towards one another, likely leading to formation of synaptic junctions. Further work would be required to determine whether true synapses had been formed at these junctions, and whether the two populations exhibit attractive cues towards one another or had 'found' each other by chance. Either way, the ability of ENSCs to integrate with SC-derived cells lends further credence to their use as a stem cell source for SCI.

2.4.3 Differentiation of ENSCs towards appropriate neuro-glial lineages

Isolated GFP+ cells and their derivatives demonstrated extensive differentiation towards neuronal lineages, as evidenced *in vitro* by TuJ1+

immunostaining of neurospheres. To provide further evidence that ENSCs could serve as a viable cell replacement source following SCI, uncultured SC and gut tissue were assessed using qRT-PCR to determine the relative expression of common nervous cell types. This confirmed common expression of neurons (TuJ1+), glia (S100+) and stem cells (Sox10+), in agreement with previous publications demonstrating the presence of these cell types in ENSC-derived neurospheres (Binder et al., 2015; Cooper et al., 2016). Expression of TuJ1 was significantly higher in uncultured SC tissue than for gut tissue. Although this seems in contradiction to published data citing equivalent numbers of neurons between the SC and ENS (Furness, 2006) at the ages examined (E14) the ENS has not yet reached its future size and complexity (Burns et al., 2002). Additionally, RNA was extracted from whole gut samples, and the levels of TuJ1 will be 'diluted' by the presence of mesenchymal tissue.

Comparisons of expression levels of uncultured SC tissue, compared to cultured ENSC neurospheres, revealed similar expression of TuJ1 and S100 in ENSC cultures compared to uncultured gut. Again, spinal cord levels of TuJ1 were significantly higher than ENSC cultures. This is likely explained by the higher levels of Sox10 in ENSC cultures compared to uncultured SC tissue, reflecting a more immature phenotype of ENSC cultures. Indeed, it has been shown that Sox10 is important in the maintenance of an enteric progenitor phenotype (Bondurand et al., 2006), and that downregulation of Sox10 coincides with increased neuronal differentiation (Young et al., 2003). Such high levels of Sox10 are therefore encouraging as a source of dividing cells for SCI.

qRT-PCR analysis of relative expression levels of genes involved in neurotransmitter production revealed the presence of all five neurotransmitters under examination in both gut and SC tissue. Levels of

nNOS, serotonin, glutamine and acetylcholine were largely similar between SC and gut tissue samples. Expression of GABA was significantly higher in SC samples compared to gut tissue. This is perhaps unsurprising, considering the role of GABA as the inhibitory neurotransmitter utilized in the CNS (Kew and Kemp, 2005). Cultured ENSCs showed largely similar expression levels to whole gut samples, with only slight decreases in the level of serotonin and glutamine in cultures ENSC samples. This is likely due to the expansion of the immature progenitor population as demonstrated by increased Sox10 expression in ENSC cultured samples compared to whole gut samples. Indeed, the culture media utilized in these studies have been previously optimized to encourage maintenance of a stem phenotype (Bondurand et al., 2003). The lack of significant difference in serotonin expression is also expected – while the vast majority of serotonin in the body is found in the gut, this is mostly produced and stored in enterochromaffin cells (Gershon, 2013), which will not have been selected during FACS isolation of ENCCs. Neural crest cell-derived neurons of the myenteric plexus produce serotonin, but in more modest quantities (Gershon and Tack, 2007).

Taken together, this data strongly supports the hypothesis that transplanted ENSCs are capable of integrating functionally into endogenous SC networks. During development, neurotransmitters play a key role both in plasticity and network formation (Herlenius and Lagercrantz, 2004). It is plausible that neurotransmitter expression in the injured environment may play a role in establishing new connections to replace severed tracts. Indeed, following injury, disruption of neurotransmitters has been implicated in SCI complications. The absence of serotonin signaling following lesioning of serotonergic tracts, for example, has been shown to result in serotonin hypersensitivity caudal to the lesion, contributing to spasticity (Husch et al., 2012). Concordantly, studies aimed at restoring levels of serotonin following

SCI have resulted in functional improvements (Antri et al., 2005; Schmidt and Jordan, 2000). These studies demonstrate the importance neurotransmitter expression holds following SCI, and highlight the importance of characterizing neurotransmitter competency of a stem cell source prior to transplantation.

2.4.4 Survival and localization of GFP+ ENSCs to the spinal cord white matter

In contrast to alternative sources of stem cells transplanted into the embryonic chick spinal cord, where transplanted cells were undetectable after several days (Prasongchean et al., 2012), we demonstrate for the first time that ENSCs, sourced from the gut, can survive in the embryonic spinal cord for up to 8 days post-transplantation. This data provides evidence of compatibility between ENSC-derived cells and a CNS environment, and supports the suitability of ENSCs for SCI repair.

2.4.5 Localization to neuronal tissue

The vast majority of transplanted GFP+ cells were found within the spinal cord and dorsal root ganglia. Very few transplanted GFP+ cells were detected outside of neuronal regions following transplantation, indicating either active ENSC localization specificity or poor survival in non-neuronal regions. The few cells found outside of TuJ1+ tissue were restricted to tissues dorsal to the spinal cord. Following transplantation of a GFP+ neurosphere into the neural tube at E1.5, the ectoderm closed over the neural tube, likely capturing some GFP+ cells. Thus the presence of these cells in mesenchymal

tissues between the dorsal spinal cord and overlying dermis were probably transplantation artifacts rather than migratory events. In support of this, GFP+ transplanted cells were not found in TuJ1- tissue ventral to the spinal cord. It is unlikely this will affect therapeutic application of ENSCs, as treatment would involve direct transplantation into the adult injury site when no such developmental morphogenic processes occur.

2.4.6 Localization to white matter

At all ages under examination, transplanted GFP+ ENSCs were found extensively within the future white matter (myelin will not begin to form until around E13 (Macklin and Weill, 1985)), and only rarely in the grey matter. Previous studies investigating transplantation of alternative stem cells into the embryonic chick neural tube have shown relatively poor localization propensities. Prasongchean *et al* demonstrated survival of rat amniotic fluid stem cells (AFSCs) for four days after transplantation into the injury site at E2.5 (Prasongchean et al., 2012), but AFSCs largely failed to home to grey or white matter, and were instead found mostly in the central canal or, more commonly, near the dorsal root ganglia, with no apparent integration. These findings contrast our observations of white matter localization following ENSC transplantation.

The results of ENSC transplantation described here are more consistent with observations by Toma *et al.*, who used the chick embryo as a model organism to assess differentiation following transplantation of induced pluripotent cell-derived motoneurons (iPSCMNs) into the developing neural tube (Toma et al., 2015). Their study revealed transplanted iPSCMNs localized to spinal cord white matter, with projections into the PNS. We observed a similar extension of our transplanted ENSCs through the dorsal

root ganglia to enter the PNS. The similarities between the current study and that of Toma *et al* demonstrates the ability of transplanted ENSCs to integrate in the spinal cord in a similar manner to transplanted motoneurons. It also suggests either a shared tendency of a percentage of transplanted cells to migrate through CNS tissue towards and into PNS tissue, or of transplanted cells being transported with surrounding tissue as development progresses and such peripheral structures are formed. Transplantation into an adult model of SCI, with examination of the extent of migration into peripheral structures, would clarify this point. In any case, the ability of transplanted cells to survive in CNS/ PNS tissues, combined with previous studies demonstrating the expression of sensory and motor neurotransmitters in cultured ENSC-derived cells (Suarez-Rodriguez and Belkind-Gerson, 2004) suggests that ENCCs may be able to re-establish sensory and motor connections.

ENSCs are derived from enteric NCCs, known to be a highly migratory population (Bronner-Fraser, 1994; McLennan et al., 2015). It was therefore plausible that transplanted ENSCs would migrate out of the spinal cord, towards tissues with a neural crest contribution. We did not observe any such spread, consistent with a recent study by Belkind-Gerson *et al*, examining the potential of ENSCs for brain injury (Belkind-Gerson et al., 2016). They demonstrated that tail vein delivery of ENSCs resulted in homing of ENSC-derived cells to the injury site and white matter of the brain. Interestingly, ENSC-derived cells were absent in other neural crest-derived tissues, including the intestines, suggesting injury as a localization cue, and implying ENSC localization to the SCI site could be similarly expected following transplantation. These results support the white matter-specific localization of ENSCs observed in our experiments, including the near-complete absence of GFP+ cells in other tissues.

Other neural crest sources have also been utilized for SCI therapy. Olfactory ensheathing glia (OEG), shown to be neural crest in origin (Barraud et al., 2010), have been transplanted into adult rodent spinal cord, resulting in functional motor recovery in 10 of 12 transplanted animals (examination of both remaining animals revealed misplaced transplants) (Keyvan-Fouladi et al., 2003). Again, consistent with our observations, predominant localization to the white matter tracts was observed, though arborisation into the adjacent grey matter also occurred. The success of OEG transplantation was attributed to their ability to survive within the CNS tissue and injury site, promoting endogenous axonal sprouting. Based on their shared ectodermal origin and similar fate-lineage, similar results could be expected following ENSC transplantations.

2.4.7 Dorsal/ ventral localization

In transplants harvested at early stages (E5.5), transplanted GFP+ ENSCs were found surrounding the entirety of the neural tube, with no apparent dorsal/ ventral preference. However, with increasing time post-transplant, ENSCs appeared to become progressively more dorsalized, resulting in almost exclusive localization of transplanted cells to the dorsal spinal cord by E9.5. In their study of iPSCMN transplantation into the chick neural tube Toma *et al* demonstrated a *mostly* dorsal localization (with more variability of grey/ white matter localization) although projections left the spinal cord by ventral roots. However, the method of inducing injury utilized by Toma *et al* was different from that used in the present study. Toma *et al* made an incision in the dorsal neural tube and subsequently implanted cells in the neural tube lumen, contrasting with our method of ablation of an entire somite-length section of the neural tube. In the stages utilised by both Toma

et al and the current study (~E3.5 and E1.5, respectively) the neural tube is entirely capable of regeneration following SCI (Shimizu et al., 1990). This has been shown to result in complete recovery of the injury site, including extension of injured axons to re-establish lost connections (Hasan et al., 1993). It is not known whether replacement of lost cells contributes to this process (Ferretti et al., 2003). However, if proliferation was indeed occurring, it is likely that this would take place towards the ventral side of the neural tube with subsequent dorsal expansion, and that relatively greater rates of proliferation would be required to enable regeneration from the method of injury used in the current study compared to that used by Toma *et al*. Replacement cells may well originate from the neural floor plate and overlying neuroepithelial cells, at least a remnant of which likely persists along the notochord following the injury method used in this study. Motoneurons of the future spinal cord originate from this region (Yamada et al., 1993), with the underlying notochord thought to induce proliferation of overlying neuroepithelial cells and differentiation of motoneurons (Hirano et al., 1996; van Straaten et al., 1985; Wilson and Maden, 2005). Further supporting a ventral-dorsal progression of regeneration, ventral neuroepithelial cells of the spinal cord produce large populations which place a pressure on the overlying dorsal cells, progressively forcing them more dorsally (Bronner-Fraser and Fraser, 1997; McConnell and Sechrist, 1980) and several such populations migrate towards the dorsal neural tube (Rowitch, 2004; Soula et al., 2001). This sequence of events likely explains the progressively dorsal localization of transplanted ENSCs.

2.4.8 Presence in injury site, bridging, A/P

At all stages examined, transplanted GFP+ cells could be found within the

injury site, an important finding if ENSCs are to serve as a cell replacement source or bridging substrate for endogenous recovery. Typically, transplanted cells were found within residual TuJ1+ neuronal tracts. Considering the majority of spinal cord injuries result in varying degrees of spared white matter tracts (James et al., 2011), such residual TuJ1 structures will likely be found in human SCI, meaning transplanted ENSCs are adhering to appropriate substrates. However, of note, some transplanted ENSCs were found within TuJ1- tissue within the injury site, demonstrating the ability to survive in areas where endogenous neurons have been lost entirely, and therefore encouraging their use as bridging structures between severed ends of the spinal cord following injury. Concordant with such use, the majority of transplanted ENSCs found in the spinal cord and TuJ1+ structures of the injury site were aligned along the anterior posterior axis. This demonstrates both the potential to integrate with endogenous networks, the vast majority of which are aligned along the anterior posterior axis, but also the ability to provide 'pathways' for endogenous neurons to follow across the injury site. Correct alignment is a target of many stem cell transplantation therapies, with some even going as far as using scaffolds to encourage longitudinal alignment and integration (Hyatt et al., 2014). A recent review defined a successful bridge as: "a multicellular structure comprised of transplanted cells that crosses the lesion site (rostrally to caudally)" (Assinck et al., 2017). Indeed, the success or failure of a particular transplantation is often determined purely by the extent of anterior posterior axonal sprouting (Lu et al., 2012). Many articles use tracing methods, both retrograde and anterograde, to discern the extent of anterior-posterior axon regeneration (Zhao et al., 2015). Thus, the common alignment along the anterior-posterior axis of transplanted ENSCs is a significant finding, and strongly advocates the potential of ENSCs to form bridges for endogenous regeneration in SCI.

Recognising the importance of cell behaviour along the anterior posterior axis, cell spread along the spinal cord was quantified in the present study. In early transplants spread along the anterior-posterior axis was relatively low, reaching ~998μm in total. A positive correlation of spread with increasing time at harvest post transplant was noticed, with spread reaching ~1600μm by E7.5 and 2687μm by E9.5, the latest time point examined in this study. This trend suggests spread may have continued in a similar manner if embryos had been left to develop longer. While it is plausible that this spread is a reflection of the overarching development of the spinal cord, and thus that transplanted ENSCs were simply 'pulled' in the direction of tissue growth, it is likely that, at least to a certain degree, this represents active processes on the part of the transplanted cells. Firstly, axonal projection was included in these measurements, known to be an active process involving extension of the growth cone and response to attractive/ repellent stimuli (Lowery and Van Vactor, 2009). Secondly, for the anterior-posterior increase in spread to be entirely related to growth a similar extension along the left-right axis of the embryo spinal cords would have been expected. If anything, we noticed a decrease in the amount of transplanted ENSCs spreading into the surrounding PNS through the dorsal root ganglia. Cell spread in other studies of stem cell transplantation varies according to the technique used. In models where an incision was made in the dorsal neural tube and a dissociated cell suspension injected into the lumen, spread was understandably greater due to passive travel along the lumen (Prasongchean et al., 2012; Zhao et al., 2013). To our knowledge, few other studies have attempted neurosphere transplantation into the developing chick neural tube. In such cases, anterior-posterior spread was not assessed (Toma et al., 2015). Thus, in comparison to injection into the lumen, it is likely that the results of our study more accurately represent migration of cells from the

transplantation site and not passive diffusion through the neural tube lumen. Thus, the current study is likely more indicative of expected results following transplantation into the adult spinal cord.

2.4.9 Differentiation of ENSCs

A logical solution to the loss of neurons during SCI would be to transplant a stem cell population capable of replacing them. However, this has not proved so straightforward. Success of mesenchymal stem cell differentiation towards a neuronal phenotype has proven inconsistent even *in vitro* (Scuteri et al., 2011). Several reports have demonstrated potential neuronal differentiation of mesenchymal stem cells following transplantation into the injured spinal cord or other CNS regions (Ryu et al., 2009; Zhao et al., 2002). However, many have questioned these reports (Qu and Zhang, 2017) and/ or failed to reproduce them or demonstrate significant neuronal differentiation of their own sources (Parr et al., 2008; Prasongchean et al., 2012). While it has been suggested that such neuronal differentiation may not be vital (Assinck et al., 2017), it is almost certainly an advantage. Previous publications have listed neuronal differentiation as the likely cause of increases in motor function following transplantation into an injured spinal cord (Abematsu et al., 2010; Lu et al., 2012).

In the current study, ENSCs readily differentiated towards a neuronal phenotype both *in vitro* and *in vivo*, following transplantation. Both immunofluorescent and qRT-PCR analysis of cultured ENSCs prior to transplantation showed a high expression of TuJ1, a pan-neuronal marker, and transplanted cells were almost exclusively TuJ1+, suggesting that these cells are potentially a good source to replace lost or injured neurons. The

results presented in the current study are consistent with those of Belkind-Gerson *et al*, who demonstrated the ability of ENSCs to survive, proliferate and differentiate towards neuro-glial lineages when transplanted into injured adult mouse brain (Belkind-Gerson et al., 2016). The discrepancy between these results of mesenchymal stem cell ENSC transplantation likely reflects an inability of the former to respond to neural cues and/ or survive in neuronal regions (Donaldson et al., 2009; Steigman and Fauza, 2007). Indeed, poor survival of MSCs has been noted before (Ruzicka et al., 2017). Differentiation of transplanted cells will undoubtedly aid in integration, and potential formation of relay circuits and lesion-spanning bridges. It would be interesting to further examine the neuronal differentiation observed in the current study to determine specific neuronal subtypes produced. However, further differentiation analysis is technically difficult due to the limited availability of antibodies raised against chick antigens. To more definitively determine the specific neuronal and glial fates of transplanted cells further novel antibodies are required, or progression to a rodent model in which such antibodies are more readily available.

2.4.10 Myelination of transplanted cells

Myelination does not occur in the chick spinal cord until E13 (Ferretti and Whalley, 2008). Consistently, processes extended by GFP+ cells in the 'injury' zone displayed the 'beaded' structure typical of unmyelinated neurons (Shepherd and Raastad, 2003), and often observed in enteric neurons (Heinicke and Kiernan, 1990). However, while enteric neurons are unmyelinated in the gut (Gershon and Rothman, 1991; Ruhl, 2006), their myelination may be possible in the adult spinal cord. Adaptation of neurons depending on their environment is a well-described phenomenon, such as

the regenerative capacity of spinal neurons when removed from the inhibitory environment of the spinal cord and placed in peripheral nerve structures (Richardson et al., 1980; Taylor et al., 2005). It is possible that enteric neurons may prove similarly adaptable and receive myelination within the spinal cord. However, even if transplanted cells prove incapable of myelination, their viability as a SCI treatment would likely be unaffected. Given the short distance required to bridge the lesion and re-connect severed tracts, the slower conduction speeds associated with non-myelinated axons (Hartline and Colman, 2007) may not impact on functional recovery. Indeed, not requiring myelination may prove advantageous in the SCI environment, in which lack of myelination and resulting Wallerian degeneration is a significant issue (Mietto et al., 2011).

2.4.11 Future work

The experiments performed in this chapter provide vital proof of principle data for the hypothesis that ENSC-derived cells can flourish in an embryonic CNS environment. The following experiments could be carried out to extend the current analysis:

2.4.11.1 Proliferation analysis

Initial high proliferation rates, which decrease with time following cell transplantation into the spinal cord, have previously been shown by Mothe *et al*, though the authors offered no explanation for this phenomenon (Mothe et al., 2008). BrdU-based proliferation assays revealed the presence of dividing cells in the transplants performed within this study, however, this proved

difficult to quantify due to a high rate of transplant death following application of BrdU. A certain amount of proliferation post-transplant would likely prove beneficial through replacement of lost neurons, but unchecked proliferation raises concern over tumour formation, a risk previous publications have taken pains to rule out (Bastidas et al., 2017; Levi et al., 2017). However, recent studies assessing NSC transplantation have noted a dose-dependent effect of transplantation on proliferation of transplanted cells, implying the ability of neural-stem cells to respond to local inhibitory cues (Piltti et al., 2015). Based on extensive similarities between CNS and ENS cells noted within this report, it is possible that ENSCs would similarly respond to local cues and restrict uncontrolled proliferation. Therefore, the limited proliferation observed in ENSC transplants within this study is likely supportive of SCI regeneration. Progression to an adult model of SCI would circumvent issues of BrdU labeling in the embryo, allowing determination of the extent of division occurring within transplanted ENSCs.

Survival of transplanted cells is also an important concern. Mothe *et al* also noted a significant reduction in the number of surviving transplanted cells in the first week post transplant (Mothe et al., 2008), and Ruzicka *et al* noted extensive death of MSCs post-transplant (Ruzicka et al., 2017). In the current study cell numbers did not appear to decrease with time following transplantation, but this will need to be assessed over a longer term. However, based on the compatibility of ENS/ CNS cells observed *in vitro*, and the initial cell spread observed, extensive death is not expected.

2.4.11.2 Assessment of functional integration

Previous work in our lab has demonstrated functional integration of ENSC-derived cells transplanted into mouse hindgut, evidenced by propagation of

calcium signaling between endogenous and grafted cells (Cooper et al., 2016) and through restoration of colonic transit (McCann et al., 2017). Though functional integration of transplanted cells may well be occurring within the chick spinal cord, as suggested by the close proximity of transplanted cells to the white matter and the extension of processes, determination of such function is technically difficult in an embryonic model. Therefore, future experiments will likely progress to an adult rodent model. This will allow electrophysiological testing/axonal tracing of transplanted cells to ascertain functional integration with endogenous cells; behavioral tests following SCI/ ENSC transplantation, assessing functional recovery; and more extensive characterization of transplanted ENSC differentiation potential, utilizing pre-existing anti-rodent antibodies.

2.4.11.3 Additional research directions under consideration

Successful future therapies will likely incorporate a combinatorial approach of cell transplantation, scaffold deposition and modification of the inhibitory microenvironment. Scaffolds facilitate cell engraftment and migration through fluid filled cavities, which otherwise offer little cell adhesion, and microenvironment modifiers offer the potential to increase survival of transplanted and endogenous cells.

Future work could therefore combine ENSC transplantation with additional therapies. Potential scaffolds include Bioglass™, which is biodegradable and has a high angiogenic potential (Gorustovich et al., 2010); and factors modifying the microenvironment are likely to include extracellular matrix modifiers such as CSPG inhibitors (reducing glial scar inhibition) (Bartus et al., 2014) or protein deiminase inhibitors (reducing calcium-related damage) (Lange et al., 2011).

2.4.12 Conclusions

These results are supportive of the hypothesis that ENSC-derived cells could be used for SCI therapy. At all ages examined following transplantation into the embryonic chick spinal cord at E1.5, ENSCs survived and differentiated towards appropriate neuronal lineages, with limited proliferation post transplant. ENSCs formed bridging connections across the injury site between the anterior and posterior spinal cord tissue, and extended axonal processes through the injury site. Transplanted ENSC cell-spread along the anterior posterior axis increased with time post-transplant. When transplanted into the injured spinal cord at E11, GFP+ ENSCs extended processes into the white matter after only 24 hours. Future studies will progress this data towards adult models of SCI. This will establish whether the ability of ENSCs to survive, proliferate and differentiate and spread within the embryonic chick can be replicated in more mature models. Advancement towards an adult model will allow the beneficial effects of ENSC transplantation on tissue regeneration post-SCI to be assessed both histologically and functionally, and provide further evidence that ENSCs can serve as a viable source of stem cells for SCI.

Chapter three: Identification of Optimal Parameters for Progression of Studies Conducted in the Chicken Embryo to an Adult Rat Spinal Cord Injury Model

3.1 Introduction

Results of the preceding studies utilizing the chicken as a model organism provide vital proof of principle data that transplanted ENSCs can survive in the spinal cord environment. However, as previously mentioned, the chicken embryo does not fully recapitulate typical SCI processes that occur in the adult human (Tator, 1995), including failure to form a glial scar (Clearwaters, 1954; Shimizu et al., 1990) or initiate an immune response (Negash et al., 2004; Seto, 1981). Therefore, there remains a need to determine whether transplanted ENSCs can survive in a 'true' injury zone (one more reflective of that observed following SCI in humans); whether this survival can continue long term; and whether such transplantation has any effect on motor recovery. This necessitates progression to a model that more accurately recapitulates the events observed following human SCI. As such models are typically more expensive than chick embryo research, a pilot study was conducted prior to initiating a large, long term and costly study of ENSC transplantation into an adult model of SCI, with the aim of identifying optimal parameters for an extended study, and providing preliminary data indicating the likely success or failure of such a project.

Rodents such as mice and rats are the most commonly used mammalian models of SCI. For the purposes of our study we selected the rat as the most suitable SCI model for progression, due to the ability of this model system to more closely mimic the SCI pathology observed in humans, including the formation of fluid-filled cavities at the site of injury. This is a key feature of the human condition, and it is thought that these fluid-filled voids may contribute significantly to the failure of axons to cross the injury site (Xia et al., 2016). The lack of supporting cells or matrices within the cavity leaves little to no substrates for cell/ axon attachment, let alone guidance/repulsion

cues that are vital for growth cone extension (McCall et al., 2012). Thus for a SCI therapy to be successfully translated from bench to bedside, it must be shown that the therapy is able to prevent, compensate for, or correct cavity formation. In the mouse, these cavities are filled with cells (Inman et al., 2002; Ma et al., 2001), thus limiting translational predictions based on data obtained from mouse models. The rat, conversely, has been well documented to form these fluid-filled cavities, providing a more accurate model for translational studies (Kjell and Olson, 2016).

As mentioned in the preceding chapter, cervical injuries are the most common in humans (Gensel et al., 2006). However, thoracic injuries are by far the more commonly studied (Sharif-Alhoseini et al., 2017), providing a greater wealth of published data. In addition, cervical injuries in the rat carry a greater mortality rate compared to thoracic injuries due to respiratory complications arising from the former, and it is generally regarded that they are less reproducible than thoracic injuries (Lane et al., 2008; Rahimi-Movaghar, 2009). For these reasons, a thoracic injury at the level of vertebrae T10 was selected.

A variety of methods can be employed to injure the spinal cord, including contusion, compression, and hemisection (Sharif-Alhoseini et al., 2017), among others. Contusions are the most established SCI model (Anderson, 1982), and with the use of equipment such as Infinite Horizon impactors, are arguably one of the most reproducible (Brösamle and Huber, 2006), allowing a more robust internal comparison within a given study. Human spinal cord injuries involve blunt force trauma, e.g. motor vehicle accidents (Nas et al., 2015), further supporting the notion that contusions more closely recapitulate human injuries (Nobunaga et al., 1999). Moreover, contusion injuries allow for graded levels of injury severity, and the accompanying pathology has been well documented (Noble and Wrathall,

1989). This permits cell transplantations to be tested in progressively more severe models, depending on the purpose of the study. As this pilot study sought to determine the optimal time of transplantation to achieve maximal cell survival a mild thoracic contusion model was utilized.

Finally, both immunocompetent and immunocompromised rodent models exist for the study of SCI. Immunosuppression can be achieved using both genetic and pharmacological means, and choice of model has significant implications for the interpretation of results (reviewed in (Anderson et al., 2011)). The future goal of ENSCs as a therapy for human SCI would theoretically involve autologous transplantation of enteric-derived cells into the SC of the same patient and so would require no immunosuppression, a key advantage of the ENS as a cell source. For the current study, however, transplants would be allogenic, involving ENS-derived cells extracted from the gut tissue of one rat being transplanted into the injured SC of another (harvesting ENS cells without sacrifice of the host is technically challenging in rodent models, due largely to the small size of the GI tract). Immune suppression was likely unnecessary for the present study, as cells for transplantation were harvested from, and transplanted into, Sprague Dawley rats to maintain strain consistency. However, Sprague Dawley rats are an immunocompetent outbred strain, and it was therefore still possible that an immune response would be mounted by the host. It was additionally possible that the GFP label could elicit an immune response (reviewed in (Ansari et al., 2016)). However, components of the immune system are likely highly valuable in the initial stages of SCI, for example for clearance (albeit incomplete) of cell debris released from necrotic or apoptotic cells (Church et al., 2017) reviewed in (Goldstein et al., 2016). In order to reduce the number of variables under examination (several immune-modulating agents are thought to have effects on cell proliferation and fate etc. (Isomoto et al.,

2007; Song et al., 2006)), and to conduct cell transplantation in a manner as close to the human reality as possible (transplanted cells in a human patient, whether autologous or otherwise, will face an activated immune system at the injury site (Fleming et al., 2006)), for this pilot study it was decided to transplant cells into an immunocompetent rat.

In summary, an immunocompetent rat model (Sprague Dawley rats – an outbred colony) with a T10 mild contusion injury was selected as the most suitable model to answer the objectives of Chapter 3, specifically, to:

- Determine early cell survival following transplantation
- Provide initial data as to suitable cell numbers for transplantation
- Identify the optimal time post-injury for transplantation
- Test injury/ regeneration quantification parameters

These results will inform the planning and execution of an extended study encompassing longer animal survival post-transplantation and behavioral assessment following injury and treatment.

3.2 Methods

3.2.1 Project responsibility disclosure

This project, using a rat injury model, involved the establishment of a collaboration with Professor Elizabeth Bradbury, Wolfson Centre for Age Related Diseases, Guy's Campus, King's College London. Professor Bradbury was approached based on her outstanding track record with regards to extracellular environment-modifying SCI therapies, and an alignment of the current study's aims with SCI models and techniques used within her laboratory. As well as providing access to the expertise of Professor Bradbury's laboratory, this collaboration provided invaluable guidance and assistance with advanced animal surgery techniques. Where methods/procedures were conducted by, or with assistance from, Professor Bradbury's laboratory, this is clearly stated (Fig. 3.1).

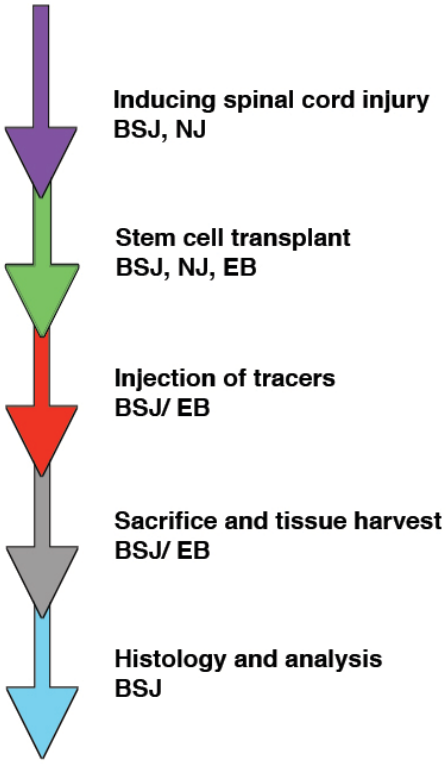

Figure 3.1: Breakdown of study contributions by collaborators at King's College London.

Contributions from members of Professor Bradbury's team is noted here. Dr Nicholas James (NJ) induced the majority of initial SCI using the impactor, and Dr Emily Burnside (EB) assisted with injection of tracers above and below the injury site, as well as with culling of the rats.

3.2.2 Harvesting of rat gut tissue

Female P7 Sprague-Dawley rats were sacrificed by cervical dislocation and death confirmed by decapitation. Following a midline dermal incision the gastrointestinal (GI) tract was harvested into Mg^{2+}/Ca^{2+} free phosphate-buffered saline (PBS) under sterile conditions. The small and large intestines were opened along the anterior posterior (AP) axis, stretched, and pinned lumen side up. The mucosal layer was peeled and removed via fine dissection. The underlying muscularis was manually chopped into small pieces (2-3mm), washed twice in PBS with centrifugation (1000rpm for 5 minutes) and incubated in collagenase (1mg mL-1) at 37°C for 15-20 minutes. Digestion was halted by addition of FACS media (DMEM F12 (Sigma Aldrich, UK), N2 (Gibco Life Technologies, UK), B27 (Gibco Life Technologies, UK), penicillin/ streptomycin (Sigma Aldrich, UK), with 2% fetal bovine serum (Sigma Aldrich, UK) and the solution passed through a pipette to encourage cell dispersal. The cellular solution was centrifuged (1000rpm for 5 minutes), re-suspended in NSM+P and passed through a 100μm cell strainer (Falcon, Corning, USA). Cells were subsequently plated at a density of ~50,000 cells mL^{-1}, onto 6 well cell culture dishes previously coated with 2% fibronectin.

3.2.3 Cell culture and FACS analysis (rat)

Cells were cultured largely according to protocols used for chick work (see Chapter 2). Notably, following initial bacterial contamination, penicillin/ streptomycin was replaced with primocin (InvivoGen, UK), a potent antibiotic and anti fungal agent, which prevented contamination in further experiments. Two days after tissue harvest, cells were collected for FACS analysis. Culture media was removed and wells washed with Mg^{2+}/Ca^{2+} free PBS. Cells were

incubated in Trypsin (1mg mL^{-1}) for 2 minutes at 37°C. Trysinisation was halted by addition of FACS media, and cells collected and centrifuged (1000rpm for five minutes). Excess media was decanted, and the resultant cell pellet re-suspended in 50µL of NSM+P. 50µL of anti-p75 FITC-conjugated primary antibody (Eurogentec) was added, gently mixed and left on ice for 1 hour. Cells were centrifuged (1000rpm for 5 min), re-suspended in 500µL fresh NSM+P (DMEM F12, N2, B27, Primocin) and FAC-sorted using a Beckman Coulter MoFlow system. Resulting cells were centrifuged (1000rpm for five minutes), re-suspended in fresh NSM+P and plated at a density of 80,000-100,000 cells per well of a 6 well dish pre-coated with 2% fibronectin. Cells were maintained by removal of 1mL media (50% total volume) and replacement with 1mL fresh NSM+P every 2-3 days. If cells became confluent or began to form neurospheres they were passaged in the same manner as chick cells (see Chapter two, Methods), in order to prevent aggregation. Prior to transplantation, cells were labeled with a self-inactivating (SIN) second generation HIV-1 based lentivirus, containing mutated Woodchuck Posttranscriptional Regulatory Element (WPRE) downstream of eGFP (Natarajan et al., 2014) to allow for post transplantation fate-mapping.

3.2.4 Animals

For this pilot study, a small number of animals (n=10) were examined, with the principle aim of finding the optimal time point for cell transplantation following contusion injury. Adult (180-200g) female Sprague-Dawley rats were used (Harlan laboratories). Female rats were selected on the premise of reduced aggression following surgery, as observed from previous investigations (unpublished), and to avoid potential gender difference

complications in subsequent analyses. 4-5 rats were housed together with cage enrichment under a 12 hour light/ dark cycle, with access to food and water *ad libitum*. All experimental procedures were carried out in accordance with the U.K. Animals (surgical Procedures) Act 1996. The studies were carried out under project license 70/832, held by Professor Stephen B McMahon, and under the personal licenses of Dr N James, Dr E Burnside and Benjamin Jevans (PIL: 17C771A1E).

3.2.5 Rat surgeries

Contusion injury was induced as previously described (James et al., 2011). Briefly, rats received perioperative analgesia (Carprofen, 5mg kg^{-1}) and were anaesthetized using ketamine (60mg kg^{-1}) and medetomidine (0.25mg kg^{-1}) by intra-peritoneal injection, and the thoracic region of their backs shaved and cleansed with iodine. Core temperature was maintained at 37°C during all procedures using a self-regulating heating blanket. All surgical tools were sterilized prior to surgery by autoclaving, and were cleaned and heat sterilized between animals. A laminectomy was performed at T10 (with the appropriate surgical site identified by the characteristic morphology of vertebrae 10), beginning with an initial incision to expose the muscle along the vertebrae. A scalpel was used to clean the overlying spinotrapezius muscle from the vertebrae, and the adjacent muscle and dermis pulled back using retractors. This musculature and the corresponding nervous tissue are associated with respiratory function, and so breathing was carefully monitored during this stage. Bone nippers were used to carefully remove a small portion of the T10 vertebrae to expose the spinal cord, ensuring the spinal cord dura remained intact (Fig. 3.2, A). Animals were carefully positioned and the spinal column stabilized using Adson forceps, and an

impactor probe of an Infinite Horizon Impactor (2.5mm tip, Precision Systems Instrumentation, Lexington, KY) was positioned 3-4mm above the exposed spinal cord (Fig. 3.2, B). This was used to deliver a controlled force trauma to the midline of the spinal cord (130 Kdyne), inducing a mild thoracic injury. Rats received two stitches to knit the spinotrapezius muscle together over the vertebrae, and sub-dermal sutures to close the initial incision. Atipamezole hydrochloride (1mg kg^{-1}), an anesthesia reversal agent, was injected sub-cutaneously, followed by 3-5mL saline solution. Rats were housed in a recovery chamber (water base thermostat 32°C, Thermocare) and carefully monitored for signs of distress over 3-4 hours. Following surgery, rats were weighed and maintained overnight in a recovery suite with softened chow, long neck water bottles and self-regulating heating blankets calibrated at 35°C. All animals received post-operative analgesia (Carprofen 5mg kg-1) the morning after surgery, and antibiotics (Baytril, 5mg kg-1, one week course).

3.2.6 ENSC transplant

Rat-derived ENSCs and their derivatives, labelled with an eGFP lentivirus as above, were prepared 2 hours before transplantation. Media was drained, cells washed with Mg^{2+}/Ca^{2+} free PBS and incubated with Trypsin (1mgmL^{-1}) for 2 minutes at 37°C. Digestion was halted by addition of FACS media, and the cells collected and concentrated by centrifugation (1000rpm for 5 minutes). Cells were re-suspended in NSM+P and counted using a hemocytometer with Alcian Blue to ensure dead cells were not included in cell counts. Prior to injection, cells were centrifuged (1000rpm for 5 minutes), and the media decanted and re-suspended in NSM+P to a concentration of 125,000 cells μL^{-1}.

Animals were randomly assigned to cell transplantation at either 3

days post-injury or 1 week post-injury (3DPI and 7DPI, respectively). For each condition, n=2 rats were harvested 24 hours post-transplantation, and n=2 rats were harvested 7 days post-transplantation. A final two rats were used as controls. 250,000 cells were injected in total per animal, 125,000 above and 125,000 below the injury zone. Rats receiving stem cell transplantations were anesthetized 3 or 7 days following contusion in the same manner as above. Wounds were reopened and the spinal cord re-exposed. Two injections (injection depth 1mm), each of 0.5µL of cell solution, were delivered at a rate of 200nL min^{-1} using an ultra micropump III (World Precisions Instrumentation, USA) immediately anterior and posterior to the injury site (Fig. 3.2, C). Wounds were then closed and animal recovery monitored as above (Fig. 3.2, D).

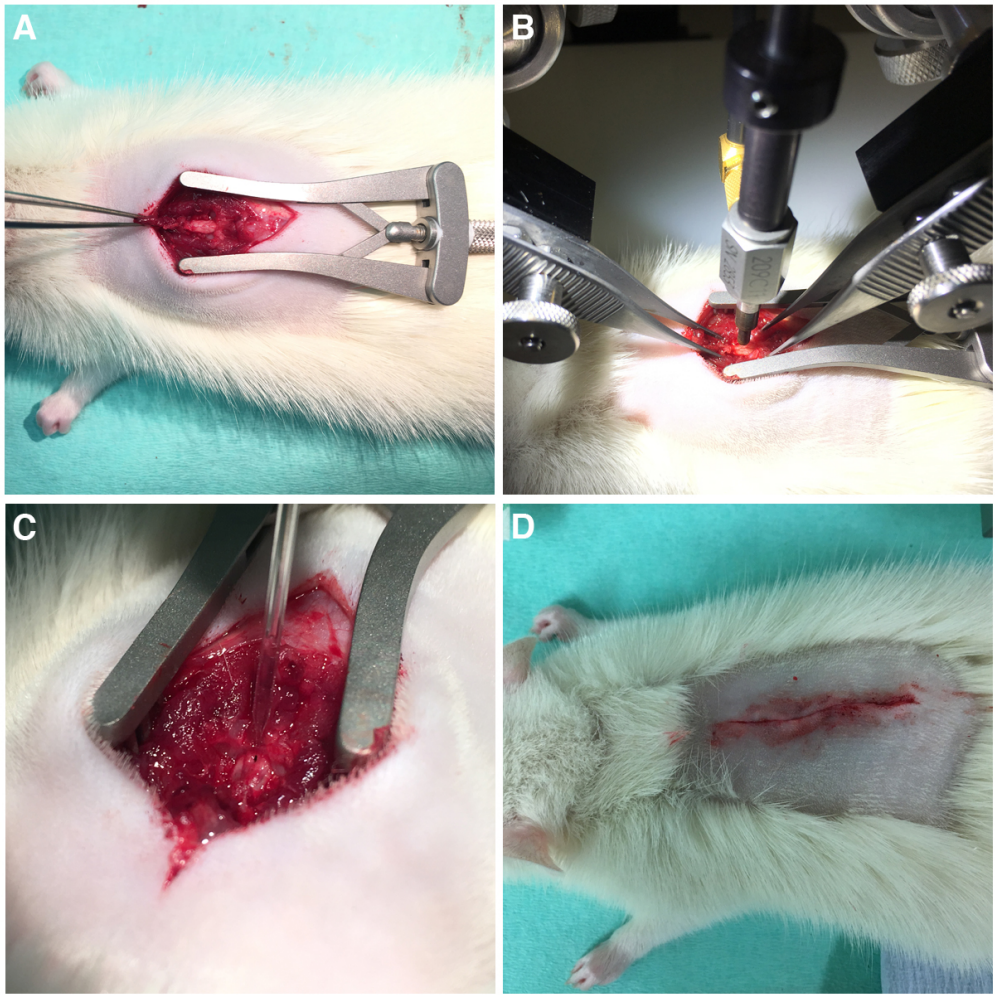

Figure 3.2: Induction of contusion SCI using an impactor probe.

180-200g Sprague-Dawley rats were anaesthetised and shaved. Following a dorsal midline incision the spinal column was cleared of musculature and a laminectomy performed to expose the SC (A). An Infinite Horizon Impactor was positioned 1mm above the cord and a controlled force impact administered to induce a contusion injury (B). The surgery site was closed and animals allowed to recover. 3 days later animals were re-anaesthetised, the surgical site re-opened and ENSCs injected into the SC (C). The overlying musculature was then sutured, and the dermis closed with subcutaneous sutures (D). All animals were monitored closely during recovery.

3.2.7 Sacrifice and tissue harvest

Rats were sacrificed via intraperitoneal injection of a lethal dose of sodium pentobarbitol (Euthatal; 80mgKg^{-1}). Once all reflexes had been lost, but just prior to heart failure, animals were fixed via cardiac perfusion. Rats were stabilized ventral side up and an A/P midline incision made to expose the ribcage. Ribs were cut at the extreme left and right of the animal and the ribcage lifted to expose the heart. A small incision was made in the right atrium. The left ventricle was then similarly opened and a perfusion catheter inserted. This was clamped in place and 0.9% saline pumped through the cardiovascular system to flush blood vessels, followed by 400mL 4% ice-cold PFA in 0.1M PBS. Pallid discoloration of the liver, toes and blood vessels lining the ribcage were used as markers of adequate perfusion. The spinal cord was harvested at the level of T10 (+/- 5mm), along with a sacral spinal cord sample, the brain, right lung, right lateral lobe of the liver, right kidney, spleen and GI tract. Samples were post-fixed at 4°C overnight and subsequently stored in PBS prior to use. Samples intended for PCR were frozen in saline at 20°C.

3.2.8 Cryosectioning

Spinal cord samples were cryoprotected overnight at 4°C in 30% sucrose and transferred to OCT (Thermo Scientific, USA). Samples were orientated for sagittal sectioning, frozen using -65°C isopentane and stored at -80°C prior to sectioning.

Frozen OCT-embedded samples were sectioned serially (20µm) using a Leica Cryostat at -22°C and slides stored at -20°C for further processing.

3.2.9 Immunostaining

This investigation was limited to assessment of spinal cord section histology and immunoflourescent analysis, sufficient to address the aims of the pilot study. Immunostaining protocols were exactly as described previously (see Chapter 2). The primary and secondary antibodies used were as follows:

Table 3.1: primary antibodies.

Protein target:	**Host species:**	**Concentration:**	**Supplier:**
TuJ1	Mouse	1:500	Covalence
GFP	Mouse	1:500	Invitrogen
GFP	Rabbit	1:500	Invitrogen
GFAP	Rabbit	1:500	Dako
Iba1	Rabbit	1:500	Wako
Ki67	Rabbit	1:500	Novocastra

Table 3.2: secondary antibodies.

Target:	Host species:	Concentration:	Supplier:	Emission wavelength:
Rabbit	Goat	1:500	Invitrogen	488
Rabbit	Goat	1:500	Invitrogen	568
Mouse	Goat	1:500	Invitrogen	568
Nuclei (DAPI)	N/A	1:1000	Sigma Aldrich	350

3.2.10 Eriochrome C staining

Eriochrome C staining was used to demarcate white and grey matter within spinal cord sections, allowing quantification of injury parameters. Serial sections of a single harvested spinal cord from each group were utilized for eriochrome C staining. Slides were thawed at RT, dehydrated in ascending EtOH series and cleared in Histochoice. Following rehydration in a reverse EtOH series, sections were stained in Eriochrome C solution (0.16% Eriochrome Cyanine-R, 0.5% sulphuric acid, 0.4% iron chloride, made up in ddH_2O). Slides were washed briefly in ddH_2O and differentiated in 0.5% aqueous ammonium hydroxide. Finally, slides were washed in ddH_2O, oven-dried at 50°C and mounted using DPX (Merck Millipore, Germany).

3.2.11 Analysis

Eriochrome C-stained sections were imaged using a Zeiss Axioplan microscope mounted with a Zeiss colour camera and analysed using Fiji (ImageJ) software. Treatment groups were randomized prior to analysis. If required, images were stitched using the MosaicJ plugin (MOSAIC Group, Center for Systems Biology Dresden (CSBD), Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany). Injury cavity area was quantified using the 'magic wand' tool, set to a tolerance of 6. Quantification began at the injury epicenter, designated as the section with the largest total cavity area, with the area of the preceding and following section also measured (+/- 20µm).

3.3 Results

3.3.1 *In vitro* characterization of ENSCs

ENSCs isolated via p75+ FACS sorting were characterized briefly prior to transplantation to ensure the correct cell types had been selected. Following isolation, cells were plated onto fibronectin-coated culture dishes and imaged 1-2 weeks later. Cultures grown for 1 week revealed numerous cells with neuronal-like morphology (Fig. 3.3, A), which began to form neurospheres after around 2 weeks in culture (Fig. 3.3, B). Cultures were fixed, and subsequent immunofluorescent investigation revealed that the majority of these cells were TuJ1+ neurons (Fig. 3.2, C), though notably, several also stained positive for the proliferation marker Ki67 (Fig. 3.2, D), indicating that a sub-population of these were still capable of division, and therefore likely maintained a stem cell state to some degree.

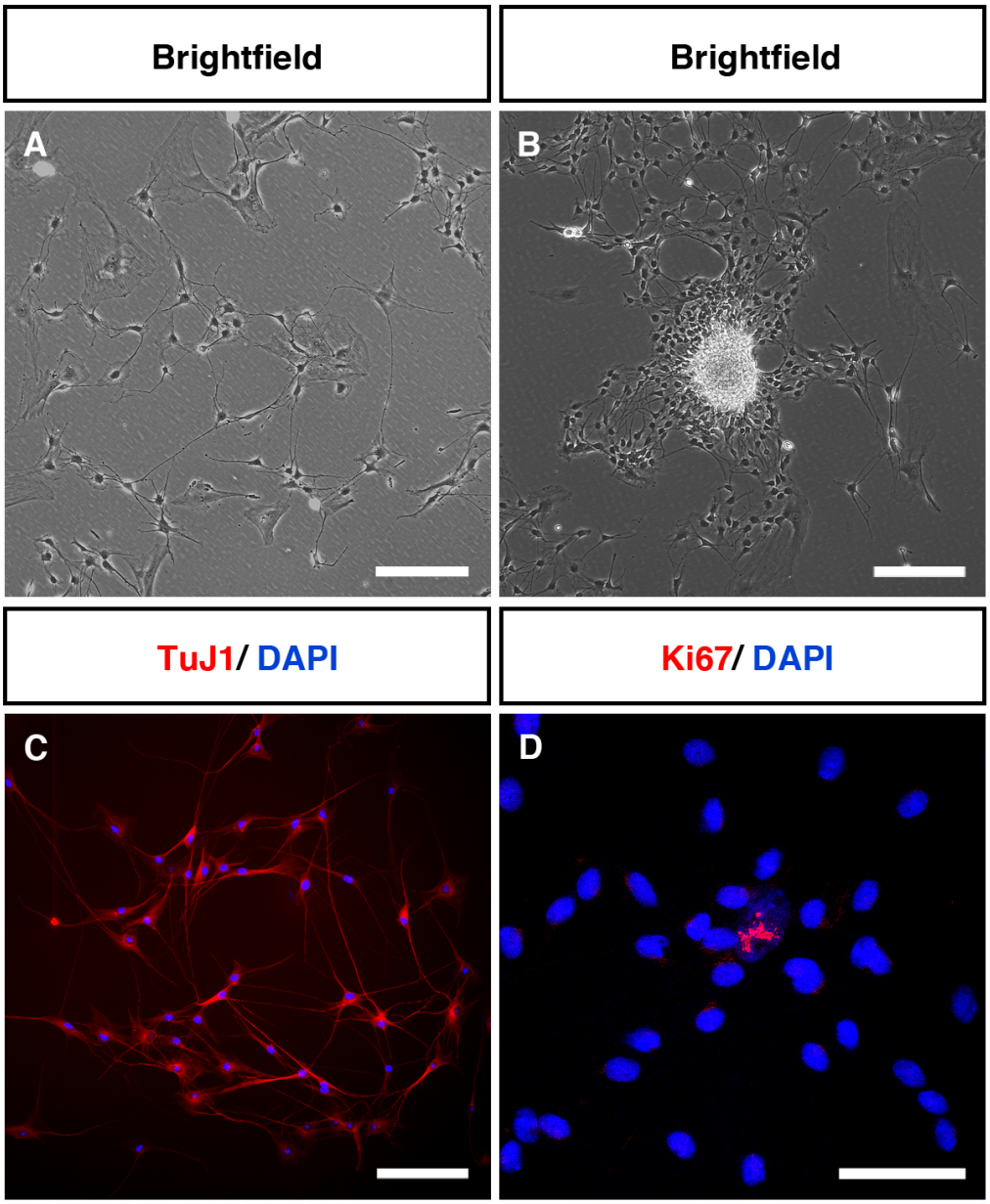

Figure 3.3: *In vitro* analysis of cell morphology/behaviour and proliferation/differentiation potential of cultured rat-derived ENSCs.

Following FACS, cell cultures display a characteristic neuronal morphology by 1 week in culture (A), and form dense neurospheres by around 2 weeks (B). The vast majority of cells stain positive for the pan-neuronal marker TuJ1 (C), indicating extensive neuronal differentiation. A small number of dividing cells was also found, as revealed by Ki67+ staining (D). Scale bar – A: 200μm, B: 100μm, C: 100μm, D: 50μm.

3.3.2 Eriochrome C staining reveals progressive cavity formation with time post-injury

More than 50% of human spinal cord injuries are classed as 'incomplete' (National Spinal Cord InjuryStatistical, 2014), referring to a degree of spared white matter. As well as facilitating varying degrees of sustained motor/ sensory function after injury, these spared tracts may provide limited structural/ biochemical substrate to support regeneration. Therefore, the ability to distinguish between grey and white matter is of key interest in tracking regeneration following spinal cord injury. Eriochrome C staining of serially sectioned spinal cords revealed clear distinction between grey and white matter (Fig. 3.4), (white matter showed intense blue staining, grey matter showed more modest blue staining), and extensive eriochrome C negative areas of neurodegeneration (Fig. 3.4, A). At the earliest stage examined (transplanted 3DPI, harvested 24 hours after transplantation) almost no cavitation was observed, whereas in all other animals (harvested at least one week post-injury) areas devoid of all staining could be detected, representing ongoing cavitation (Fig. 3.4, B, C, D). Injured tissue (demarcated by eriochrome C negative areas of tissue), and in later stage animals, cavities (demarcated by total absence of tissue, Fig. 3.4, B, C, D, arrows), appeared to extend across both grey and white matter. The largest cavity size (1577.2 μm^2) was observed in the rat transplanted 7DPI and harvested 24hours later. In comparison, the cavity size of the rat transplanted 7DPI and harvested 7 days later was diminished (626.9 μm^2).

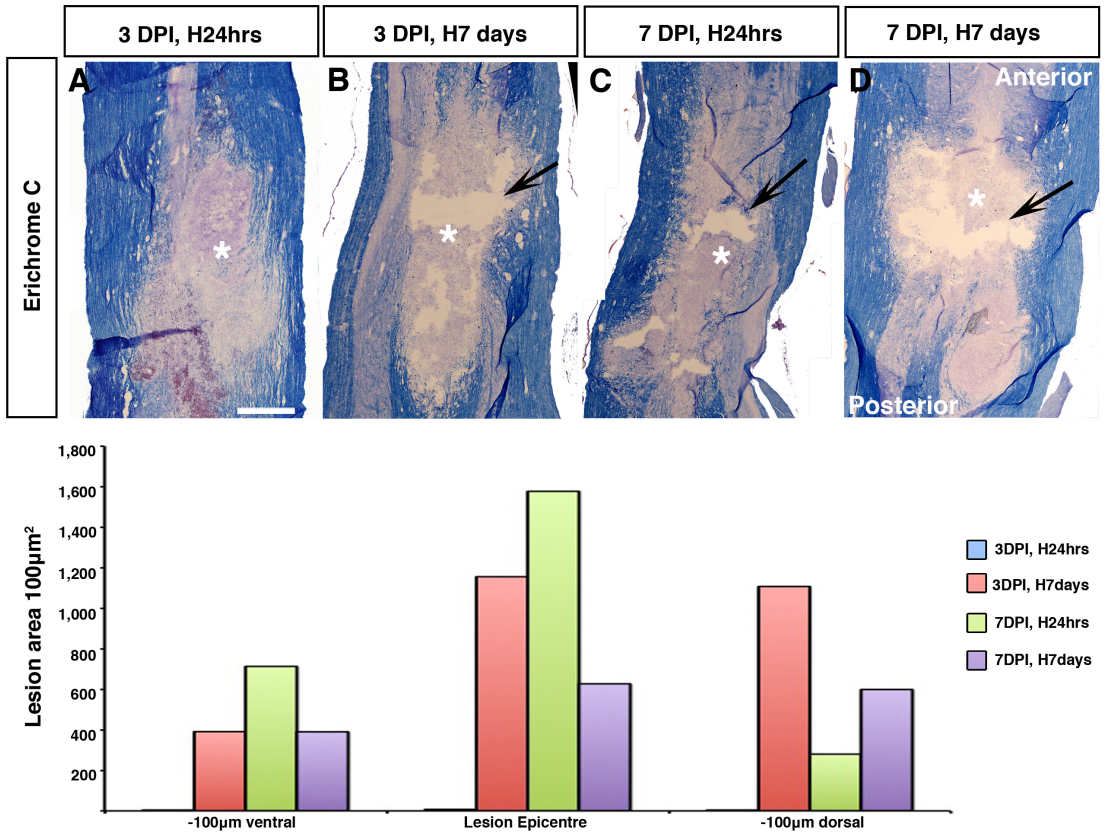

Figure 3.4: Histological comparison of SCI histology between controls and animals transplanted with ENSCs.

Eriochrome C staining revealed injured tissue in all animals examined (injury zone demarcated by *), with regions of spared white matter seen in all groups. Cavities could be seen in all animals harvested >1 week post-injury (B, C, D, arrows). Quantification of the total cavity area revealed the largest cavities in animals transplanted 1 week post-injury and harvested 24 hours later. Animals transplanted at the same stage but harvested 1 week later showed decreased cavity area. Scale bar 100µm, n=1.

3.3.3 Transplanted ENSCs survive within the spinal cord, and differentiate towards a neuronal lineage

Spinal cord injury severely disrupts normal tissue architecture, including

severing of neuronal tracts and destruction of large areas of neuronal cell bodies. Serial sections of harvested spinal cord tissue were stained with a panel of antibodies to ascertain both the effect of injury on tissue architecture, and the survival of ENSCs post-transplantation. Antibody staining with TuJ1, a pan-neuronal marker, revealed clear distinction of grey and white matter, and clearly demarcated damaged tissue around the lesion (Fig. 3.5). TuJ1+ staining was observed throughout the cord, with more intense staining of the white matter, including multiple linear A/P fibres. Regions of TuJ1- staining were observed within the center of the cord, in regions of close proximity to the overlying bruise observed at the time of harvest (Fig. 3.5). These regions contained extensive DAPI+ nuclei within these TuJ1- zones, demonstrating the presence of cells within the injury zone at this stage (Fig. 3.5, A, B, D, asterisk. However, these did not stain positive for any markers examined.

At all time points examined, numerous transplanted GFP+ cells were found within the spinal cord (Fig. 3.5). Cells displayed a neuronal morphology, and area of GFP+ signal did not appear to obviously diminish with extended time post transplant. However, cell localization varied dramatically depending on time of transplant post injury. Cells transplanted three days post-injury and harvested 24 hours later were found within the injury zone, occupying what would presumably have been the lesion cavity, or surrounding the injury zone in a 'halo' (Fig. 3.5, A). Spinal cord tissue of animals transplanted 3 days post-injury and harvested 7 days later revealed transplanted GFP+ cells forming apparent bridging structures between the anterior and posterior ends of the injury zone (Fig. 3.5, B). Cells transplanted 7 days post-injury, however, were either found anterior to the injury zone, in small, distinct clusters, or, in one case, sparsely scattered around the dorsal extremities of the spinal cord (Fig. 3.5, C, D). No obvious changes in

transplanted cell localization were observed between tissue harvested 24 hours or seven days after transplantation. Transplanted cells appeared to remain connected to one another, with very few ENSCs found in isolation. Transplanted GFP+ cells did not occupy the entirety of the lesion site, with significant areas of the lesion site occupied by DAPI+/TuJ1-/GFP- tissue (Fig. 3.5, C). At all time points examined, TuJ1+/ GFP+ cells could be observed, implying appropriate neuronal differentiation (Fig. 3.6, B), although it is unknown if this differentiation occurred before or after transplantation. Additionally, GFP+ cells appeared to align with neuronal tracts, most clearly seen in the spinal cord periphery (Fig. 3.6, B, C). Finally, GFP+ cells were invariably found continuous with endogenous TuJ1 staining within the injury zone, possibly implying reciprocal benefit towards survival. In support of this, TuJ1+/GFP- staining was very rarely found within the injury zone (Fig. 3.6, B).

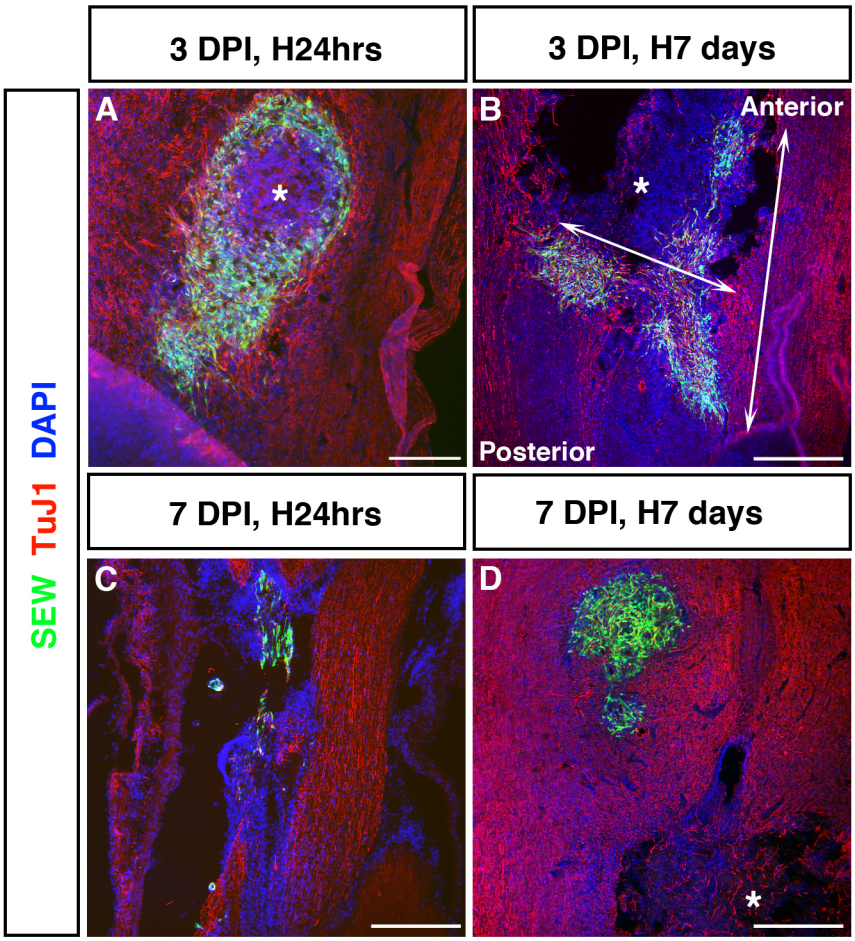

Figure 3.5: Comparison of ENSC survival/localization in the injured rat SC across the four transplantation time points examined.

Immunostaining of serial spinal cord sections with the pan neuronal marker TuJ1 (red) reveals the SCI zone (demarcated with an *) and surviving transplanted cells. A - GFP+ cells transplanted three days post-injury surround the spinal cord injury zone of rats harvested after 24 hours, concentrated at the posterior end. B - spinal cord tissue of rats transplanted three days post-injury, and harvested 7 days post-transplantation. GFP+ ENSCs form bridging connections through the injury zone. C - spinal cord sections of rats transplanted seven days post-injury and harvested 24 hours after transplantation. D – spinal cord sections taken from rats transplanted seven days post injury and harvested 7 days post transplantation. Scale bars: A - 200μm, B, C, D - 500μm.

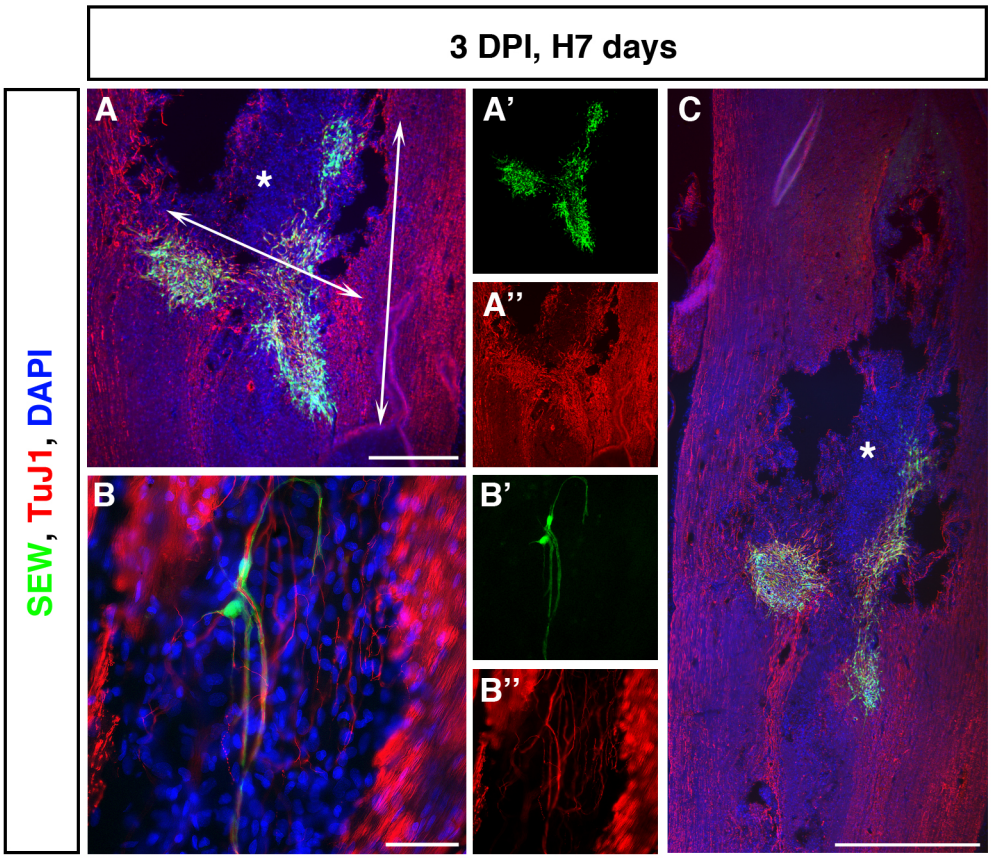

Figure 3.6: Immunofluorescent assessment of differentiation and localization of transplanted ENSCs within the injury zone.

A – GFP+ transplanted cells formed a continuous bridge of cells crossing the injury zone. These areas stained positive for TuJ1. Co-labelling was confirmed at higher magnification, demonstrating GFP+/TuJ1+ cell bodies and axons (B). Collated tile scan images reveal the full extent of tissue injury (TuJ1- regions), including initial cavity formation and the degree of spreading from transplantation site. Scale bar – A, 200μm, B, 50μm C, 1mm.

3.3.4 Transplanted cells do not exacerbate astrocytic activation, and cross the glial scar

Typically following SCI, astrocytes are recruited to the injury site, forming a

dense astroglial scar around its periphery which is thought to present both a physical (Moeendarbary et al., 2017) and chemical (Silver and Miller, 2004) barrier to regeneration. To determine the effect of this glial activation on transplanted cells, spinal cord sections were analysed by immunofluorescence. GFAP staining was detected throughout the spinal cord, with greater staining in the white matter (Fig. 3.7, A). In naïve, uninjured animals (harvested 7 days after transplant), transplanted cells were found with slightly elevated endogenous GFAP+ staining around the transplant periphery (Fig. 3.7, A). This staining appeared comparable (if slightly more advanced) to glial activation surrounding the injury zone of animals harvested 24 hours after injection 3DPI (Fig. 3.7, B). In animals harvested at least seven days post-injury, GFAP+ staining appeared more intense, and was concentrated around the injury zone (Fig. 3.7, C). Interestingly, in later stage transplants where GFP+ cells were not found within or crossing the injury zone (injury zone demarcated by asterisks), GFAP staining revealed no concentration around the transplanted GFP+ cells, implying that the cells themselves do not cause a glial reaction (Fig. 3.7, D, E). Concordantly, GFAP staining did not co-localise with transplanted cells: no co-labelled cells were found, implying that transplanted ENSCs do not differentiate towards an astrocytic lineage (Fig. 3.7, C). When found within the injury zone, GFP+ cells were located within, but distinct from, the glial scar; and when found crossing the injury zone, GFP+ cells were similarly distinct from GFAP+ staining, appearing to have either found or caused a breach in the scar (Fig. 3.7, E).

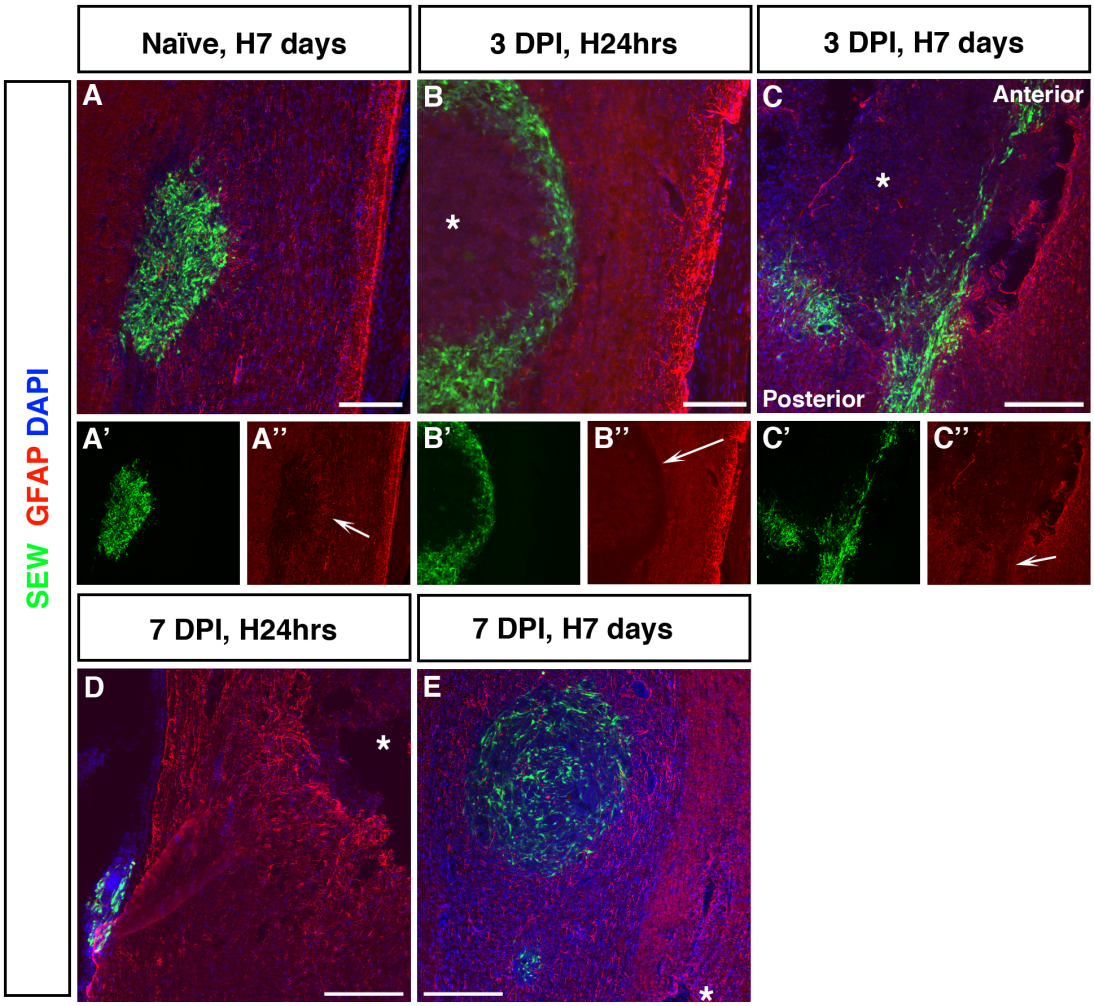
Naïve, H7 days
3 DPI, H24hrs
3 DPI, H7 days
SEW GFAP DAPI
A
B
C
Anterior
Posterior
A'
A''
B'
B''
C'
C''
7 DPI, H24hrs
7 DPI, H7 days
D
E

Figure 3.7: Assessment of the colocalization of transplanted ENSCs with the glial scar.

Immunostaining of serial spinal cord sections with the astrocytic marker GFAP. Cryosections of naïve (uninjured) animals revealed GFAP staining throughout the spinal cord. Slight elevation was seen around the transplant periphery (A'', arrow). B - in rats transplanted three days post-injury and harvested 24 hours later, GFAP+ staining did not overlap with GFP+ cells (B'', arrow). C - spinal cord tissue of rats transplanted 3 days post-injury and harvested after 7 days post-transplant displayed more intense GFAP staining around the border of the injury zone (injury zone demarcated with an *). GFP cells cross the glial scar (C'', arrow). D - spinal cord tissue of rats transplanted 7 days post-injury and harvested 24 hours later show intense GFAP+ staining around the injury zone periphery, but little GFAP+ signal around transplanted cells. E - spinal cord tissue of rats transplanted 7 days post-injury and harvested 7 days later does not appear to show increased GFAP+ staining around transplanted cells, and no GFAP+/ GFP+ cells. Scale bars –200μm.

3.3.5 Transplanted cells survive in the injury zone despite presence of activated macrophages, and do not exacerbate an Iba1+ immune response

Immune infiltration is a major complicating factor of SCI, with the lesion site typically being occupied rapidly by invading macrophages and neutrophils (Carlson et al., 1998). Indeed, the presence of these cells is often linked to cavity formation (Mietto et al., 2015). To determine the effect of stem cell transplantation on immune activation around the injury site, and vice versa, harvested spinal cord tissue was stained for Iba1, a macrophage marker. Positive staining was revealed weakly (although distinctly) throughout the cord, presumably demarcating microglia (Fig. 3.8, D, arrow). In cryosections

of naïve (uninjured) animals, Iba1+ staining was detected at the transplantation site (Fig. 3.8, A). Staining was intense within the injury zone of all animals under examination, revealing numerous Iba1+ microglia both within the future lesion cavity and surrounding the scar (Fig. 3.8, B, C, E). The staining intensity appeared to partially diminish with time post-injury (Fig 3.8, E, compare staining around transplanted cells with Fig. 3.8, A). Interestingly, these Iba1+ cells did not overlap with areas of GFP+ staining in the injury zone. Indeed, in cases where GFP+ cells were found *within* the injury zone, Iba1+ cells were found surrounding, but never continuous with, transplanted cells, possibly implying that transplanted ENSCs can survive in the presence of Iba1+ cells (Fig. 3.8, B). Similarly, when GFP+ cells were observed crossing the injury zone, they appeared to either cause, or exploit an existing gap between the surrounding Iba1+ cells (Fig. 3.8, C). Of note, in later stage transplants where GFP+ cells were found adjacent to the injury zone, Iba1+ staining was not found to be more intense around transplanted cells than in the rest of the cord, implying that, similar to GFAP staining, the presence of transplanted cells *per se* does not cause an immune reaction (Fig. 3.8, E, compare staining indicated by arrow and arrowhead).

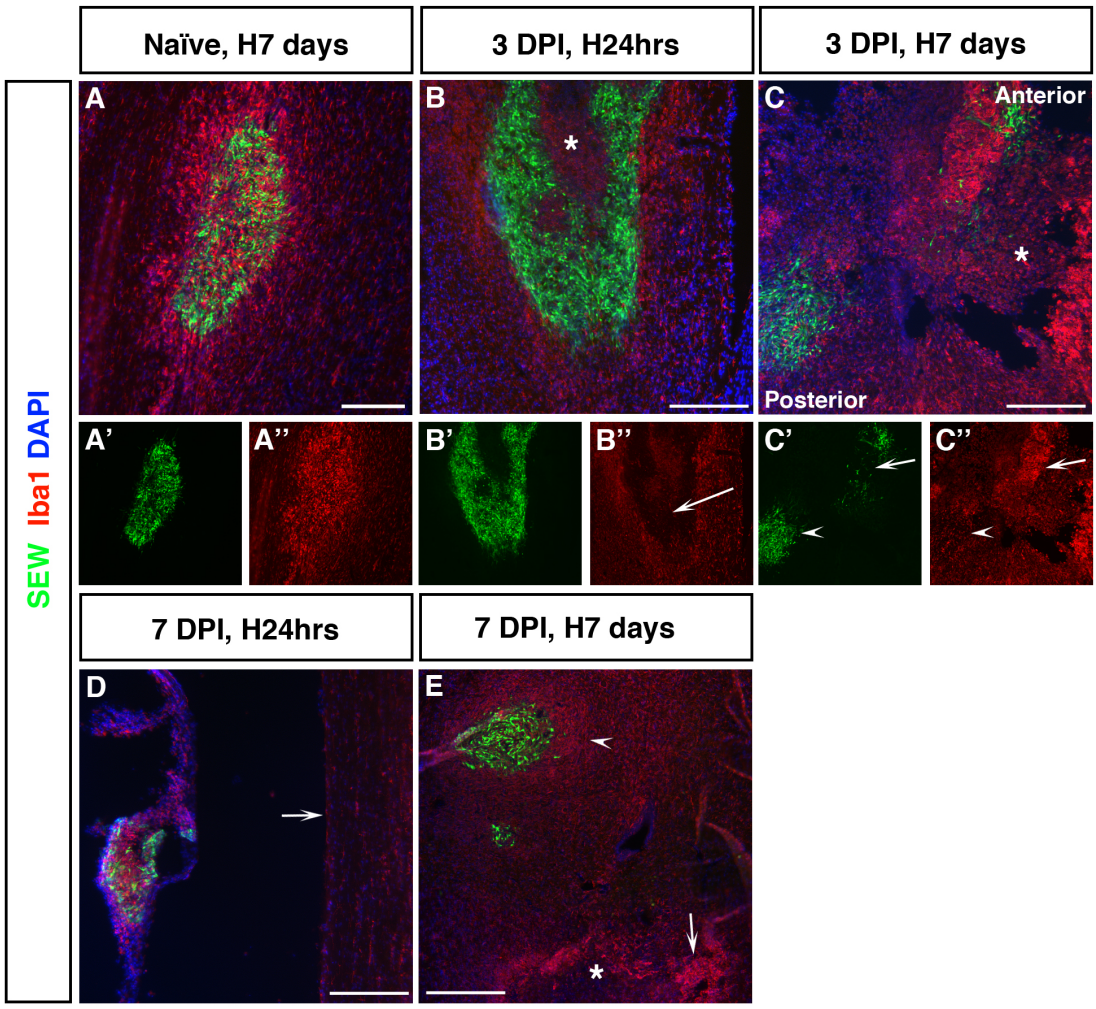
Naïve, H7 days
3 DPI, H24hrs
3 DPI, H7 days
SEW Iba1 DAPI
A
B
C
Anterior
Posterior
A'
A''
B'
B''
C'
C''
7 DPI, H24hrs
7 DPI, H7 days
D
E

Figure 3.8: Assessment of the degree of immune cell activation in response to ENSC transplantation.

Throughout the spinal cord, low levels of Iba1+ ramified cells can be seen, with concentrated Iba1+ staining within the spinal cord injury zone (asterisks) revealing extensive macrophage-based immune activation, usually demonstrating a rounded morphology. A – cryosections of naïve (uninjured) animals reveal Iba1+ cells throughout the cord, displaying a ramified morphology. Iba1+ staining is increased in areas of transplanted cells. B – tissue harvested 4 days after injury, before cavities have completely formed, Iba1+ cells can be seen extensively within and around the injury zone. Iba1+ cells do not appear to occupy the same space as transplanted cells (arrow, A''). C – tissue harvested 7 days after transplantation at 3DPI reveal the beginnings of cavity formation, with dense clusters of Iba1+ cells prolific throughout the injury zone. Iba1+ cells (arrow) did not consistently surround GFP+ transplanted cells (arrowhead). D – GFP+ transplanted cells survived distal to transplantation site, despite presence of activated Iba1+ cells. E – tissue harvested 7 days after transplantation 7DPI, in which GFP+ cells were found anterior to the injury zone, revealed intense Iba1+ staining around the injury zone, but did not show similar staining surrounding transplanted GFP+ cells. Scale bar 500μm.

3.4 Discussion

3.4.1 Summary

The contusion rat model utilized here resulted in a clear injury zone as demarcated by eriochrome C and TuJ1 staining, which revealed both early cavity formation and surrounding injured tissue. Although tissue within the injury zone was TuJ1- (likely reflecting neurodegeneration) with a surrounding glial 'scar' and Iba1+ inflammation, DAPI staining revealed the presence of cell nuclei within the injury zone, indicating cavity formation was poorly advanced. Such absence of cavitation is to be expected following a mild contusion injury (Cheriyan et al., 2014), and is in accordance with study design and aims. Therefore this study demonstrates for the first time the ability of transplanted ENSCs to survive within the SCI zone of adult rats, differentiate towards an appropriate lineage, and potentially integrate with endogenous cells to form bridges across the injury zone. With this important information, we subsequently progressed to a larger study, as described in Chapter 4.

3.4.2 Eriochrome C staining allowed for quantification of lesion histology

Here we sought in part to identify appropriate analyses upon which to base a larger study. Erichrome C staining was selected to determine clear demarcation of the grey and white matter, and to allow quantification of a range of injury markers, including cavity size. While this pilot consisted of an n of 2, therefore ruling out statistical analysis of the results, the data collected clearly showed that the methods selected could allow robust quantification

providing greater experimental replicates. In particular, cavity size was seen to vary between groups. The decrease in cavity size of rats transplanted 7DPI and harvested 7 days later, compared to rats transplanted 7DPI and harvested 24 hours later may reflect a neuroprotective effect of transplanted cells. However, this could also simply reflect variability between animals. Greater n numbers will be required to address such potential for variability.

3.4.3 The developing glial scar restricts ENSCs from the injury site

As well as the simple question of whether transplanted ENSCs survive in the spinal cord, this study revealed important information regarding the behaviour of transplanted cells within the injured spinal cord. Cells transplanted at 3 days post injury appeared to localize within the injury zone, possibly forming bridging structures across the injury, aligned along the anterior-posterior axis, with increasing length of time between cell transplantation and tissue harvest. Cells transplanted 1 week post injury, however, were found adjacent to the injury zone or scattered sparsely in dorsal-most tissue sections. This discrepancy is perhaps most likely attributable to injury zone maturation. The vast majority of stem cell transplantations into the injured spinal cord have been targeted at the acute, rather than chronic phase of spinal cord injury, and it has largely been found that transplantations within the acute time frame are able to induce motor recovery, an effect not usually observed following chronic transplantations (Cusimano et al., 2012). While the chronic phase is typically designated as weeks after the initial injury (Kjell and Olson, 2016), this is an arbitrary designation, with the injury process likely taking place as a graded progression. With increasing time post injury, exponential numbers of astrocytes are recruited/ produced at the injury zone, with glial scar formation

beginning between 1-2 weeks after injury (Kjell and Olson, 2016; Loy et al., 2002). As numbers of these cells grow, so does the concentration of inhibitory molecules they produce, such as chondroitin sulphate proteoglycans (CSPGs) (Morgenstern et al., 2002). Additional cells are also recruited to the scar, including fibroblasts (Fawcett and Asher, 1999). Cell degradation continues to occur within the injury zone, scattering cell debris, including myelin degradation products, which are detectable in the rat spinal cord around 7 days post-injury (Yang et al., 2017). Finally, immune infiltration is known to undergo 'peaks' during the transition from acute to chronic stages, including a particular increase in neuroinflammation at around 7 days post-injury in rats (Beck et al., 2010). Thus, as time progresses post injury the injury zone becomes a progressively more toxic environment, and forms a greater barrier in terms of both physical and chemical properties. It is probable that this accumulation of cell-toxic material is responsible for the inability of cells transplanted at later stages to enter, occupy or bridge the lesion. It is also possible that with increasing time post injury, the worsening 'bruise' seen on the dorsal side of the spinal cord makes targeting of cell transplantation more difficult, possibly resulting in injections more distally both anterior and posteriorly than would be optimal, meaning transplanted cells would have further to migrate to reach the injury zone.

Importantly, the vast majority of transplanted cells were found in, or adjacent to, the injury zone. Very few cells were found more diffusely throughout the spinal cord, and such cells were usually in sections immediately before or after the injury zone. In the case of transplants 7 days post-injury, small clusters of cells were found on the spinal cord periphery. In addition to demonstrating that there may be a 'window' of transplantation efficacy before this time, this potentially demonstrates that cells not actively engaged in the injury zone are either passively forced out of the lesion site,

or actively leave it and survive in more favorable conditions. Therefore, any extended study should look carefully for undesirable cell spread both within, and external to, the spinal cord.

3.4.4 Transplanted ENSCs form bridging structures

In addition to providing preliminary data to determine the optimal time of transplant, this pilot study yielded many findings encouraging a more extensive study, and highlighting the value of conducting behavioral studies in such an extended scenario. Firstly, the differentiation of transplanted cells towards a neuronal lineage; the propensity of these cells to align with endogenous TuJ1+ tracts where present; and the formation of anterior-posterior structures through the injury zone, heavily implies the potential of transplanted cells to form 'bridges' for regenerating axons to follow. Previous studies have extensively demonstrated that such cell bridges can allow the regeneration of axons across the lesion site, often resulting in an improvement in motor ability post-injury (Ziegler et al., 2011). Following transplantation at 3 days post-injury, both rats harvested at 24 hours post-transplantation revealed GFP+ transplanted cells localized to the centre of the injury zone, while both rats harvested 7 days post-transplantation showed cells aligned along the anterior-posterior axis, with cells appearing to cross the injury zone boundary to reach spared endogenous tissue. With such small 'n' numbers, and based on such a short time frame between harvest points, although it is difficult to say conclusively, it is possible that transplanted cells are extending further throughout the injury zone with time. It would be interesting to see whether this trend continues, and to what extent, during an extended study. The lack of significant numbers of isolated GFP+ cells implies that transplanted ENSCs are maintaining a behavior observed during

ENS development. During development ENCCs are seen to largely maintain physical connections with other ENCCs as they migrate (Young et al., 2014). Cells that become detached usually 'wander' - decreasing in migration speed relative to 'connected' migrating ENCCs, until the migration wave front catches up with them. As well as demonstrating that migration within spinal cord tissue, as opposed to the mesenchymal-derived tissue these cells have evolved to respond to, does not seem to affect their usual behaviour, this provides further evidence that these cells would be able to form an intact bridging structure for axonal regeneration across the injury zone.

In this study, the fate of transplanted cells was limited to TuJ1. However, based on initial *in vitro* results demonstrating the presence of dividing ENSCs, it would be beneficial in future studies, to determine firstly, if any cells continued dividing following transplantation, and secondly, the fate of any such cells, including differentiation towards particular neuronal sub-populations, such as motor or sensory neurons.

3.4.5 ENSCs are able to cross the glial scar, and did not differentiate towards a glial phenotype

A key result highlighted by the present study was the low astrocytic response to transplanted cells. Cryosections taken from rats transplanted 3 days after injury show cells within the injury zone, surrounded by GFAP+ endogenous tissue. However, as highlighted by cryosections of rats transplanted 7 days after injury, where transplanted cells were found excluded from the injury zone, no obvious increase in GFAP+ staining was observed surrounding transplanted cells compared with adjacent tissue. This suggests that transplanted cells within the injury zone were not causing/ exacerbating the endogenous glial reaction. While slightly elevated GFAP+

staining was observed surrounding transplanted cells in naïve (uninjured) animals, this staining intensity appeared reduced compared to that seen surrounding the injury zone of injured animals. It is therefore possible that this lower astrocytic activation was caused by damage resulting from the act of transplantation itself. However, this will have to be examined in greater detail to determine conclusively the effect of transplanted cells on astrogliosis.

This study also found several instances of transplanted cells that appeared able to cross the glial scar (as demarcated by GFAP staining) to reach spared spinal cord tissue. It is possible that transplanted cells were only exploiting pre-existing 'gaps' in the surrounding GFAP+ cell scar, in which case it will need to be determined whether these 'opportunistic bridges' are maintained long enough for endogenous axonal recovery along them. If transplanted cells were actively able to cause glial scar breaches, then this would be also be a key finding - the glial scar presents both a physical and chemical barrier to regeneration (Silver and Miller, 2004). If transplanted ENSCs are able to effectively 'ignore' this limitation it may dramatically increase the likelihood of endogenous axonal regeneration across the injury zone. Previous reports documenting the ability of transplanted cells to cross the glial scar have also detected endogenous axonal regeneration through transplanted cell bridges (Ramon-Cueto et al., 2000).

In addition to effects on endogenous astrocytic activation, the possibility of transplanted cells themselves differentiating towards an astrocytic lineage, and therefore exacerbating SCI pathology must be examined. Previously published data has shown that transplanted cells often differentiate towards a glial lineage, with some even going as far as to suggest that astrocytic differentiation may be the default pathway following transplantation into the injured spinal cord, potentially contributing to glial

scar formation (Kang et al., 2012; Nakamura and Okano, 2013). In this pilot study, it was found that transplanted cells do not appear to differentiate to an astrocytic-like lineage, as indicated by an absence of GFP+/GFAP+ cells. Therefore, it is unlikely that transplanted cells are contributing to any significant degree to the development of the glial scar.

Given that two of three requirements for a cell-based bridge strategy are the ability to integrate into endogenous tissue, and to not stimulate a glial scar reaction (Fawcett, 2008), the importance of transplanted ENSCs 'crossing' the scar while apparently causing no obvious increase in GFAP+ staining (including a lack of transplanted cell differentiation towards an astrocytic lineage) cannot be underestimated. An extended study could provide an ideal setting to assess this ability, and possibly test methods to enhance glial scar breaching. Double labelling with GFAP and TuJ1, in addition to GFP labelled transplanted cells, would reveal both scar breaching/exploitation, provide further data supporting the lack of scar-reaction to transplanted cells, and demonstrate endogenous or transplant-derived axonal regeneration across the scar.

3.4.6 ENSCs did not enhance the immune response, as detected by Iba1

This study utilized immunocompetent rats to receive allogenic cell transplantation. A particularly interesting finding, therefore, was the lack of increased macrophage activation or proliferation detected by Iba1 immunostaining following cell transplantation in injured animals. Similar to detection of astrocytic reaction in response to cell transplantation, the lack of increased Iba1+ staining surrounding transplanted cells located *outside* of the injury zone, compared to the Iba1+ staining surrounding transplanted cells *within* the injury zone, suggests that the transplanted cells themselves

are not causing an overt immune reaction. Additionally, levels of Iba1+ staining within the injury zone of rats transplanted 3DPI (where transplanted cells were found within the injury zone) appeared similar to levels of Iba1+ staining within the injury zone of rats transplanted 7DPI (where transplanted cells were found outside of the injury zone). This suggests that levels of Iba1+ cells within the injury zone are not increased in the presence of transplanted ENSCs. It must be noted that in naïve (uninjured) animals, transplanted cells were found surrounded Iba1+ cells. It is very possible, therefore, that at least initially, ENSCs do cause an immune response. However, the intermingling of Iba1+ cells and transplanted cells was not observed in animals transplanted following injury. Since naïve animals were harvested 7 days post-transplant, it is possible that the Iba1+ cells surrounding transplanted ENSCs represent early migration of macrophages/microglia to the 'injury' caused by cell transplantation. The lack of such intense Iba1+ staining surrounding transplanted cells found apart from the injury zone at the later time point of 2 weeks following transplantation suggests that the Iba1+ response seen in naïve animals may be a transient effect. Nonetheless, this will need to be addressed in further detail.

It is also important to remember that Iba1 does not detect the entirety of the immune response, and that other components, such as T cells are known to be involved in the immune response following SCI (Beck et al., 2010; Gattlen et al., 2016; Satzer et al., 2015). These and other components of the immune system would need to be examined before concluding that transplanted cells do not elicit an overt immune response.

Even so, the lack of a strong Iba1+ response to transplanted cells in injured animals, and the observation that transplanted cells are able to survive in an immunocompetent animal, is encouraging. If, after a more thorough evaluation of additional branches of the immune response,

transplanted cells are found not to increase the injury zone immune reaction, it may reduce/avoid the necessity of immunosuppressant treatment for patients receiving ENSC transplants, which would be receiving the less immunogenic autologous transplants. This will need to be assessed in greater detail following an extended study. This is of particular importance when considering previous reports of enhanced survival of transplanted cells following immunosuppression (Khankan et al., 2016), possibly suggesting a rejection of transplanted cells using allogenic transplants. Additionally, both the lack of increased Iba1+ staining within the injury zone of animals with transplanted cells found within the injury zone compared to those with transplanted cells outside of the injury zone, and the observation that cells appeared able to survive in the presence of Iba1+ cells, encourages further testing of the ability of transplanted ENSCs to survive in/modulate the immune response of immunocompetent animals.

3.4.7 Conclusions

This pilot study reveals that transplanted ENSCs possess many properties that will likely prove advantageous for SCI recovery. Transplanted cells survived within, and formed bridging structures through, the hostile environment of the SCI zone. GFP+ staining within the injury zone overlapped with endogenous TuJ1+/GFP- staining, implying a possible synergistic relationship encouraging survival through possible formation of 'bridges'. Concordantly, transplanted ENSCs appeared to cross the glial scar, and co-labelled for TuJ1, demonstrating differentiation to appropriate lineage. Finally, transplanted ENSCs did not seem to cause overtly increased activation or proliferation of astrocytes or macrophages/ microglia.

These findings support an extended, long-term rat study of ENSC

transplantation into the spinal cord following injury. Further, they provide key insights into the planning of such a study. Cells survive transplantation, and based on the findings of this pilot study, transplantation 3 days post-injury appears to yield the desired outcome of finding cells within the injury zone, either occupying the lesion cavity or crossing the scar. This time point will therefore be utilized in further studies. As ENSCs survived in the spinal cord despite the presence of Iba1+ cells, immunocompetent Sprague Dawley rats will continue to be used. Additionally, based on the incomplete occupation of the injury zone by transplanted cells, it would be advantageous to increase the number of transplanted cells to achieve the best possible chance of observing a positive effect on locomotor recovery. As mentioned previously, the contusion model selected allows for graded injury severities. As expected in animals suffering a mild contusion, rats in this study developed very mild cystic cavitation, which proved quantifiable through erichrome C staining and area thresholding using Fiji software. With the knowledge that transplanted ENSCs can survive in a SCI environment, we can now progress to a more severe, cavity-causing contusion. Finally, the presence of a select few clusters of transplanted cells, particularly in tissue harvested from spinal cord transplanted seven days post-injury, reveals the need to assess cell spread both within the spinal cord, and to external tissue sites, providing important information as to the safety of ENSC transplantation.

Chapter four – An Histological and Behavioural Assessment of the Therapeutic Benefit of Enteric Nervous System Stem Cell Transplantations into the Injured Rat Spinal Cord

4.1 Introduction

Results of the preceding chapter demonstrate that ENSCs transplanted into the SC following a mild contusion injury survive up to 2 weeks post-transplantation. This is an encouraging basis for a larger study. Despite their paracrine benefits towards endogenous recovery, survival of mesenchymal stem cells following transplantation into the injured spinal cord has typically been poor, with few cells found up to one month after transplantation (Arai et al., 2016; Boido et al., 2014; Ruzicka et al., 2017). In contrast, NSCs have been shown to survive for extended periods of time post-transplantation (Dulin and Lu, 2014; Ide and Kanekiyo, 2016). Such long-term survival of grafted cells may well represent a key factor in stem cell-based SCI recovery. Axonal regeneration speed has been shown to vary across various anatomical domains of the central nervous system, with regeneration through the dorsal root averaging 2.1+/-0.5mm day^{-1} (Wujek and Lasek, 1983). In support of this finding, many studies did not observe any difference in functional outcomes before 3 weeks post-transplantation, possibly reflecting the time needed for axons to extend far enough to impact on functional improvement (Kadoya et al., 2016; Keirstead et al., 2005; Pan et al., 2008). Typically, such studies noticed small changes after the first few weeks, which increased progressively with time. Although the extensive survival of ENSCs post-transplantation observed in the previous chapter is encouraging, there is a need to determine whether transplanted ENSCs would survive for a similar time to that seen following NSC transplantations, sufficient to aid endogenous recovery. Additionally, the previous results were obtained from a mild contusive injury. As many patients suffer more traumatic injuries, it was decided that it would be advantageous to determine if transplanted ENSCs were able to survive in a more severe contusive injury model. This would also

allow behavioural testing to be conducted in a more relevant model to human SCI.

Stem cell therapy-based therapies for SCI holds great promise in terms of cell replacement (Mothe et al., 2008; Ogawa et al., 2002), potential modification of the inhibitory micro-environment (Kadoya et al., 2016) and endogenous neuroprotection (Mitsui et al., 2005). Indeed, several clinical trials have demonstrated the safety of stem cell transplantation into the injured spinal cord (Mendonca et al., 2014; Satti et al., 2016) and many are currently ongoing, assessing, amongst other things, the functional benefits of transplantation. However, while the promising results of numerous studies encourage further research into their application, no single stem cell treatment has resulted in complete, and permanent reversal of paralysis. This suggests the need for additional studies, including combinatorial interventions.

Extensive work has investigated the utility of a vast array of potential treatments, including both stem cell-alternatives and stem cell-enhancers. *In vitro* manipulation of stem cells prior to transplantation has been suggested, and the potential to direct stem cells down particular lineages prior to transplantation has been demonstrated (Hu and Zhang, 2009; Nistor et al., 2005). Increasing neuronal differentiation may prove particularly important considering differentiation down certain astrocytic lineages has been associated with increased allodynia (Klein and Svendsen, 2005). Alternatively, numerous studies have shown significant functional benefits resulting from stem cell-alternatives, such as modification of the microenvironment. This has included both manipulation of the immune response (Lee et al., 2003) and extracellular matrix (Stichel et al., 1999). Such modifications may support survival of transplanted cells by reducing the difficulty they face to engraft. Many such therapies could potentially boost the success of stem cell

transplantations. Considering the vast majority of transplanted ENSCs differentiated preferentially towards a neuronal rather than astrocytic lineage in the previous two chapters, modification of the extracellular environment, as opposed to *in vitro* 'priming', was deemed a more appropriate target for this study.

A large hurdle for axons crossing the injury site, and almost certainly for stem cells transplanted into the injury site, are the chondroitin sulphate proteoglycans (CSPGs). CSPGs consist of a core glycoprotein with variable glycosaminoglycan side chains (Kwok et al., 2012; Siebert et al., 2014). At least nine CSPG groups have been identified to date, including the common extracellular matrix components aggrecan and brevican (Bandtlow and Zimmermann, 2000). Indeed, most CSPGs are extracellular, and are secreted to exert their functions outside of the cell (Horii-Hayashi et al., 2015). While they form components of some tissue types, such as cartilage (Brown and Eames, 2016), they are predominantly known for their roles in cell adhesion, growth and migration (Silver and Silver, 2014; Yuan et al., 2016a). Of key importance, CSPGs are known to play a significant role in neural development through their effects on axon growth and guidance (Wiese and Faissner, 2015), and in neural health in adulthood through modulation of neural plasticity and synapses (Frischknecht and Seidenbecher, 2008; Miao et al., 2014).

CSPGs are known to have roles in CNS repair following damage such as stroke (Liu et al., 2014), and have been demonstrated within the SCI zone (Asher et al., 2001). More specifically, CSPGs are found in high concentrations within the glial scar surrounding a CNS lesion (McKeon et al., 1991), and indeed, extensive evidence has revealed their secretion by astrocytes following activation (Ohtake and Li, 2015; Yu et al., 2012). CSPGs have been known to inhibit axon growth for over twenty years (Grumet et al.,

1993). More recently, this has been shown to be due to an immobilization of the growth cone (Silver and Silver, 2014). As stated above, CSPGs have an established role in restricting plasticity and maintaining existing circuits in the adult CNS. When CSPG expression is increased in the SC lesion the innate growth retarding properties of the CSPGs actively inhibit axonal recovery (Bradbury et al., 2002). This is largely believed to be due to their usual action becoming a hindrance (Bartus et al., 2012), as increased plasticity following injury would presumably aid in re-establishing lost circuitry.

Several groups have shown the growth retarding effect of CSPGs as well as the significant improvement in axon re-growth, lesion cavity-reduction and indeed, functional gains following CSPG degradation (James et al., 2015; Moon et al., 2001). In particular, degradation of CSPGs has focussed on injection of the bacteria-derived enzyme chondroitinase ABC (ChABC). Early work demonstrated the ability of ChABC to increase axon growth *in vitro*, in SC explants taken from injured animals (McKeon et al., 1995). Following this, the ChABC gene was selectively cloned into plasmids and lentiviruses for injection into the injured SC (Bartus et al., 2014; Muir et al., 2010). Viral ChABC injections into the spinal cord following injury have resulted in a marked decrease in cavity size, increased axonal regeneration (see Fig. 4.1 for proposed mechanism) across the lesion and a functional improvement in locomotor abilities. These results are incredibly encouraging, and demonstrate the importance of such chemical modulators of the injury zone. Subsequently, the ability of ChABC to break down CSPGs in vitro has been demonstrated (Crespo et al., 2007). In this study, cells transfected with the ChABC-containing lentivirus demonstrated the ability to synthesize and excrete the ChABC protein to degrade extracellular CSPGs.

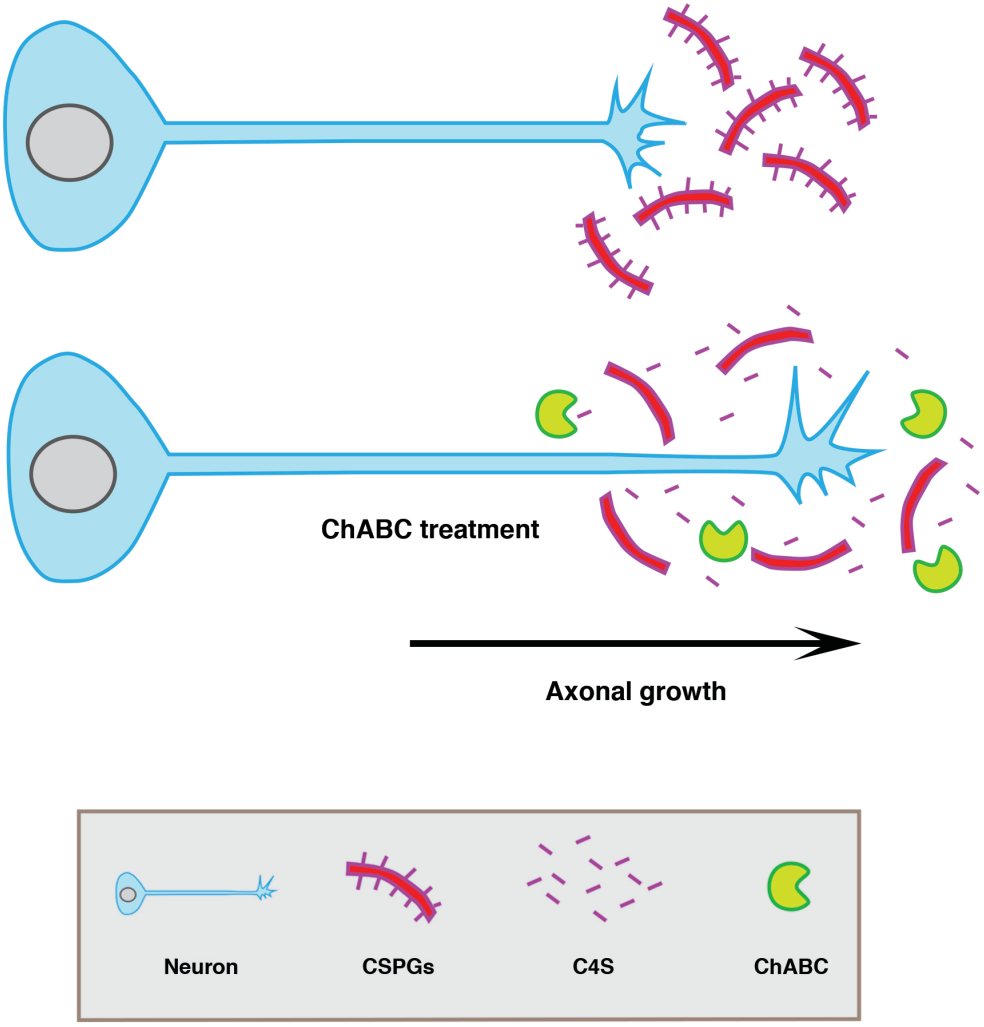

Figure 4.1: Proposed mechanism of axonal extension following ChABC treatment.

Endogenous axons are unable to re-cross the injury site following injury, at least in part because of the accumulation of inhibitory CSPGs within the glial scar. CSPGs consist of a proteoglycan core with multiple chondroitin sulphate (CS) side chains. ChABC, a lysase with broad specificity, degrades glycosaminoglycans such as CSPGs to disaccharides. Products of this digestion include C4S. Such degradation removes the inhibitory properties of CSPGs, allowing axons to extend through the glial scar (Prabhakar et al., 2005a; Prabhakar et al., 2005b).

Here it is proposed that co-application of a CSPG inhibitor with stem cell transplantation will increase cell engraftment and encourage increased bridging, and thinning of, the glial scar, thereby allowing enhanced axonal regeneration across the lesion. CSPGs have been previously documented to act as inhibitors of neural crest migration, and thus nullifying their effects will likely result in increased spreading of transplanted ENSCs across the injury site (Henderson and Copp, 1997; Perris and Johansson, 1990). Other studies have shown the multiplicative, rather than additive, effect of combining treatments, demonstrating the benefit of combinatorial therapies (Sarveazad et al., 2017).

The parameters used to gauge the success of a given study are crucially important, and should be tailored accordingly for separate studies. In addition to unique desired experimental outcomes, specific desires of patients should be addressed. Considering that the most often-cited demand of SCI patients is regaining some form of motor function, behavioural assessments are as crucial towards demarcating the successes of a project as a histological examination (Anderson, 2004). Ladder testing, involving navigation of a horizontal ladder with unevenly spaced rungs, has been extensively used to assess functional improvements in the motor ability of rats following SCI and subsequent treatment (Bartus et al., 2014). However, while informative, functional tests such as the ladder test are often not sensitive enough to highlight subtle alterations in function arising from sparing of white matter tracts or axonal re-growth following stem cell-derived bridge formation. For this purpose, tracing can be a powerful tool. Both retrograde and anterograde tracing has been used to evaluate increases in sensory and motor re-growth, respectively (Hou et al., 2016; Li et al., 2016a; Steward and Willenberg, 2017).

The experiments in this chapter build upon studies in the preceding chapter, including progression to a more severe injury type more representative of a typical human SCI; extended survival of animals post-transplant; testing of a combined ChABC-based viral treatment with stem cell transplant; the use of behavioural tests to assess motor improvements; and the use of injected tracers to determine extent of axonal bridging across the injury zone.

4.2 Methods

4.2.1 Animals

As in the previous chapter, adult (180-200g) female Sprague-Dawley rats were used (Harlan laboratories). 4-5 rats were housed together with cage enrichment under a 12 hour light/ dark cycle, with access to food and water *ad libitum*. All experimental procedures were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986.

Behavioral testing and histological analyses were conducted while blinded to the treatment groups. Randomization assigning animals to treatment groups was conducted by a collaborator whom, following the initial injury stages, played no further part in the investigation. A total of 26 animals were used, with n=6 assigned to each of 4 groups (contusion, no treatment; contusion, cell transplantation only; contusion, cell transplantation and lentiviral injection; contusion, lentiviral injection only) and an additional n=2 for the control group. All surviving animals were sacrificed at 16 weeks post injury via anaesthetic overdose and cardiac perfusion as per Chapter 3 (Fig. 4.2). For tracing experiments, n=3 per group were used.

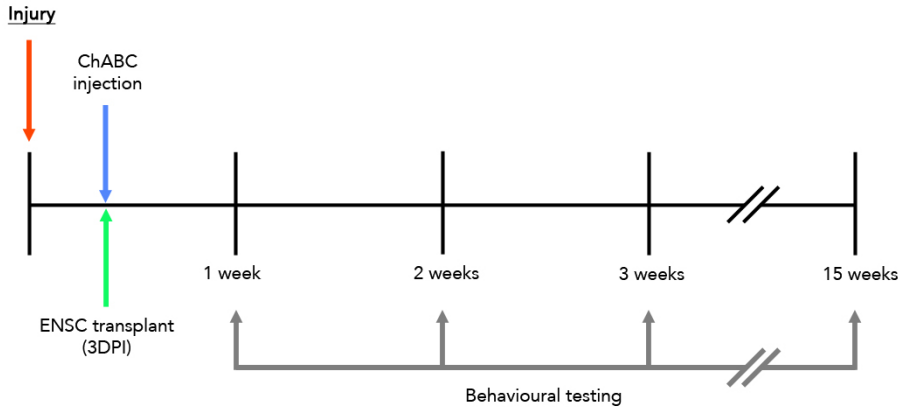

Figure 4.2: Study timeline.

From induction of contusion injury at day 0, the entire study was completed in 16 weeks. Following post-operative care after the initial injury, rats were re-anaesthetised 3 days post injury (DPI) and injected with either ENSCs, ChABC, or both. 4 days later behavioural testing commenced, and was thence repeated weekly until 15 weeks. The following week all animals were sacrificed via anaesthetic overdose and cardiac perfusion as per Chapter 3.

4.2.2 Chondroitinase lentivirus

The ChABC gene derived from Proteus vulgaris was modified to include mammalian preferred codons in place of five cryptic N-glycosylation sites, and an additional mammalian signal sequence (Muir et al., 2010). This was sub-cloned into a lentiviral transfer vector driven by the mouse phosphoglycerate kinase promoter (LV-ChABC, (Bartus et al., 2014) to create an integrating but self-inactivating vector, pseudotyped with VSV-G. Viral particles were concentrated to 1.03^{10}GC (genome copies) mL-1 by ultracentrifugation and titration via p24 antigen mediated ELISA (lentivirus made by Professor Joost Verhaagen (Netherlands Institute for Neuroscience).

4.2.3 Rat contusion injury surgery

Contusion injuries were conducted as described in Chapter 3. Injury severity was increased from mild to moderate by increasing the force applied by the impactor probe of an Infinite Horizon Impactor (Precision Systems Instrumentation, Lexington, KY) from 130kdyne to 150kdyne.

4.2.4 Injections of viral ChABC

Rats undergoing LV-ChABC (see above) treatment received intraspinal injections (injection depth 1mm) immediately anterior and posterior of the injury site prior to closing. 0.5µL of LV-ChABC was delivered at a rate of 200nL min^{-1} using an ultra micropump III (World Precisions Instrumentation, Europe). Wounds were then closed and animal recovery monitored as above.

4.2.5 Behavioural analysis

The horizontal ladder test was used as a measure of locomotor ability/ recovery (Fig. 4.3). Prior to surgery animals were trained on the task to provide baseline data. Starting one week after surgery, testing was then conducted on a weekly basis until completion of the study by an examiner blinded to the treatment groups. Rats were placed individually onto a horizontal ladder 1m in length, with irregularly placed rungs. Rats were encouraged to cross the ladder by offering Sucrose/ peanut butter pellets at opposing ends. Testing was captured using a Sony DCR-SX30E Handycam,

and data was extracted from slow-motion reviews of complete runs made from right to left, left to right, and right to left. The number of correct foot placements and foot 'slips' was counted for each run and the number of correct placements taken as a percentage of the total number. These scores were then averaged across the three runs to provide the final score.

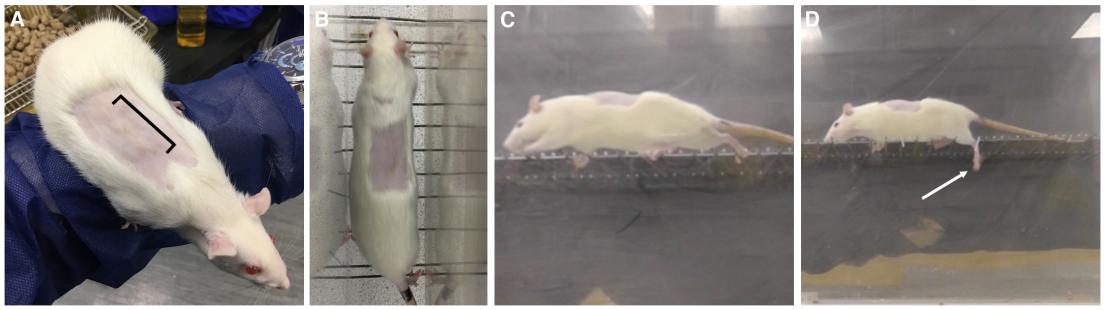

Figure 4.3: Motor recovery following SCI assessed using the horizontal ladder test.

Within 2-3 weeks after injury, the surgery site is barely visible (A). Starting 1 week post-injury and transplantation, performance was measured by the ability of animals to traverse the horizontal ladder. This consisted of two parallel, transparent Plexiglas walls placed approximately 6 cm apart, to prevent animals turning round (B). A series of unevenly spaced rungs were placed between the walls, parallel to the floor, providing a horizontal ladder for the animals to cross (C). Rats were recorded traversing the ladder right to left, left to right, and right to left again, and the results averaged. The percentage of correct foot slips was recorded, with hind paws slipping between the rungs (D, arrow) taken as a 'foot slip'.

4.2.6 Axon tracing

To determine the extent of axon regeneration across the injury site both retrograde and anterograde tracing was utilized. One week prior to study

completion, n=3 of each group were randomly selected. Animals were anesthetized and a T12 laminectomy performed as above. A simultaneous C2 laminectomy was performed using the exact same methodology. The anterograde tracer Fluorogold was used to label anterior-posterior regeneration, administered using a midline intraspinal injection (injection depth 1mm) of Fluorogold at C2 at a rate of 200nL min-1 using an ultra micropump III (World Precisions Instrumentation, Europe). Biotinylated dextrine amine (BDA) was then administered via an intraspinal injection in the same manner at T10. Wounds were closed as above, with identical surgical recovery procedures.

4.2.7 Cell survival/ spread quantification

Serial sections of the spinal cord of stem cell-transplanted rats harvested 16 weeks post-injury were assessed to determine the survival of transplanted cells and corresponding spread. GFP antibody-labeled sections were imaged using a Zeiss Axioplan microscope mounted with a Zeiss colour camera and analysed using Fiji (ImageJ) software. Treatment groups were randomized prior to analysis. Images were stitched using the MosaicJ plugin (MOSAIC Group, Center for Systems Biology Dresden (CSBD), Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany). The number of GFP+ cells was quantified using FIJI software. The *subtract background* process was utilized, with a *rolling ball* of 10 pixels, and subsequent application of a *median filter* set to a 1 pixel radius. Cell number was then determined by the find *maxima function*, with a noise tolerance of 544. The amount of GFP+ pixels was quantified using the 'Threshold' tool, set to a tolerance of between 44 and 255 (measurements limited to threshold). Quantification began at the first section to contain positive GFP

signal, and moved sagittally until the last section containing GFP signal. Each section with GFP+ signal was analysed and pooled to give a total, summed intensity. For anterior/ posterior and dorsal/ ventral spread, the section with the greatest spread was chosen for each rat, and the extent of spread determined as the farthest GFP+ signal (cells or projections) in either direction.

4.2.8 PCR detection of transplanted cell spread

Cell spread to tissues other than those transplanted into is a potential risk following stem cell transplantation. To determine whether such spread occurred following ENSC transplantation into the injured spinal cord, samples from peripheral organs were analysed by PCR to detect GFP. Post-mortem organs harvested from transplanted rats included the right medial lobe of the liver; spleen; right kidney; and left lung. 10 samples (~30mg) were collected at random from each organ for further processing. Cryosections of transplanted animals confirmed to contain transplanted GFP+ cells by fluorescent microscopy were used as a positive control. Positive sections were recovered and genomic DNA extracted in the same was as peripheral organ samples.

4.2.8.1 Genomic DNA extraction

~30mg of tissue was dried at RT. Tissue was suspended in 25µL proteinase K solution (1µL proteinase K (Sigma Aldrich, UK), 40µL DNA extraction lysis buffer (100mM Tris.Cl, 5mM EDTA, 0.2%SDS, 200mM NaCl, pH8)). Thermocycling protocol used was as follows:

Table 4.1: Genomic DNA thermocycling programme

Step	Temp °C	Time
1	55	4 hours
2	85	10 minutes

4.2.8.2 Precipitation of genomic DNA

Cellular debris and other impurities were removed from genomic DNA using salt precipitation. 1µL from each of the 10 samples collected from each organ were pooled, and this was diluted in3M Na-Acetate and 100% EtOH to a final concentration of 0.3M NA-Acetate and 70% EtOH. Samples were incubated on ice for 30 min and centrifuged at 14,000g for 30 min at 4°C. The supernatant was discarded and the pellet washed in 70% EtOH. Sample was centrifuged at 14,000g for 15 min at 4°C, the supernatant discarded and the pellet re-suspended in 10µL ddH_20.

4.2.8.3 PCR detection of GFP

Table 4.2: PCR primers

Probe target	Primer sequence	Product size	Tm
GFP	F: CACATGAAGCAGCACGACTT R: TCCTTGAAGTCGATGCCCTT	167	59.13 59.02
GAPDH	F: GTTGTGGATCTGACATGCCG R: GGTGGAAGAATGGGAGTTGC	171	59.27 58.82

1μL of precipitated DNA solution was used for each PCR reaction. Reaction volumes, components and concentrations were as for chapter 2.

Table 4.3 Chapter 4 PCR cycling programme

Step	Temp °C	Time
1	94	3 minutes
2	94	30 seconds
3	58	45 seconds
4; go to step 2, 35 cycles	72	30 seconds
5	72	2 minutes
6	4	Hold

PCR products were analysed by agarose gel electrophoresis. 10μL product was loaded into a 2.5% agarose gel (diluted in TAE buffer, with Ethidium bromide for visualization), at 160mV for 45 minutes.

4.2.9 Statistical analysis

Except where noted, all analyses were conducted on at least 3 individual animals from each group, selected at random by an investigator blinded to the treatment groups. Eriochrome C data were analysed by one-way ANOVA. Transplanted cell spread and survival, and endogenous retrograde axon regeneration were analysed using Student's t test (two-tailed). GraphPad Prism software was used for all statistical analyses. p values of <0.05 were taken as significant. All error bars represent standard error.

4.3 Results

4.3.1 Transplanted ENSCs survive transplantation, and ChABC lentivirus injection resulted in breakdown of CSPGs

Following tissue harvest of spinal cords at week 16 post-injury, success of both ENSC transplantation and injection of the ChABC lentivirus were confirmed by immunofluorescence. Digestion of CSPGs by the bacterial enzyme ChABC was assessed by detection of C4S, a product of ChABC-mediated degradation of CSPGs. ENSC transplantation and survival was confirmed by immunostaining with an anti-GFP antibody.

Several animals from all four groups were selected in a blinded fashion and analysed to ensure results were representative. In both the ENSC + ChABC and ChABC-only groups, C4S+ staining was visible throughout much of the spinal cord, appearing concentrated around the lesion cavity (Fig. 4.4, A and C). The ENSC-only, and no treatment groups, showed no positive staining (Fig. 4.4, B and D) (background staining detected in several samples appears to be fluorogold 'bleeding' through the channels, Fig. 4.4, D). In samples treated with both ENSCs and ChABC, transplanted cells co-localized with areas of CSPG+ staining, potentially implying that transplanted cells themselves had been transduced, or at least that ChABC expressed by transduced, endogenous cells was able to reach areas rich in transplanted cells.

Numerous GFP+ ENSCs were found within the spinal cord. These were mostly located at the anterior edge of the lesion, though substantial spread towards the posterior limit of the lesion through both dorsal and ventral spared tissue was frequently observed (in some animals, simultaneous

spread both ventral *and* dorsal to the lesion was noted). Transplanted ENSCs were almost invariably found in one or more large clusters, and very rarely found as solitary cells. Extensive integration appeared to have occurred, with cells found aligned along the A/P axis of endogenous tracts. In several animals, transplanted ENSCs were observed to cross the injury zone through a dorsal-ventral 'bridge' which bisected the injury zone (Fig. 4.4, C).

Transplanted ENSCs exhibited relatively small, polar cell bodies, and extended extensive processes. These projected both towards neighboring ENSCs and into the surrounding SC tissue. In several instances, single axons were found projecting rostrally from the transplant site for several millimeters. The vast majority of ENSCs were TuJ1+, implying the potential for integration with SC tissue. Indeed, comparison of corresponding GFP and eriochrome C sections revealed that areas of densely populating ENSCs also stained positive for eriochrome C, implying that these transplanted cells had been myelinated.

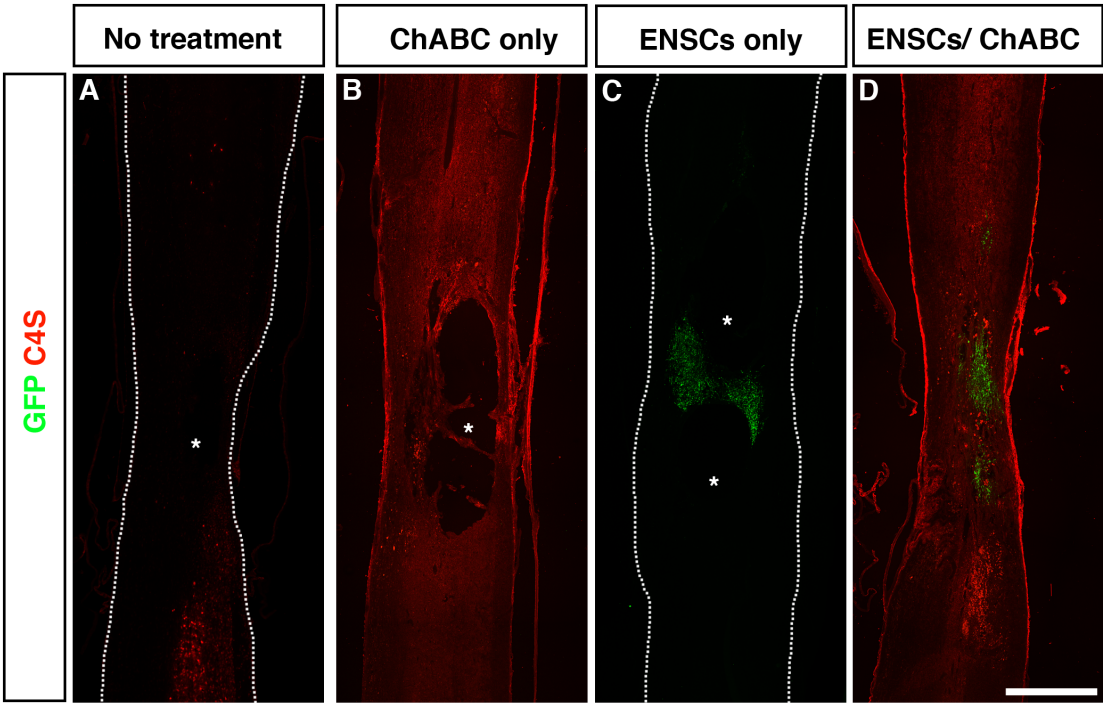

Figure 4.4: Immunostaining used to determine success of ChABC injection and stem cell transplantation.

Longitudinal cryosections of spinal cords harvested at 16-weeks post-injury were analysed for markers indicative of successful treatment. Breakdown products of CSPGs, including chondroitin-4-sulfate (C4S) disaccharides, are released following ChABC degradation, and can be detected by immunofluorescent staining (B, D). Transplanted ENSCs can be visualised within the spinal cord injury zone (C, D). Asterisks indicate lesion cavity. Scale bar 1mm.

4.3.2 Combined ENSC and ChABC treatment significantly improves lesion histology

The pathology of the spinal cord following injury has been well characterised (Tator, 1995). In particular, a central, fluid-filled cavity and surrounding preserved tissue are central features. To assess whether stem cell transplantation and/or ChABC therapy had any effect on these markers

following insult, serial sections were stained with eriochrome C and the area of the cavity quantified. Results were statistically analysed using one-way ANOVA.

Eriochrome C staining revealed typical SCI cavities in all rats (Fig. 4.5, A-D). Quantification of the cavity area revealed a decrease in the average total cavity size in rats treated with ChABC alone, but this was not significant (16.29 vs 16.47mm^2, p=>0.999). Transplantation with stem cells alone resulted in a decrease in cavity area compared to non-treated animals, but this was not significant (11.85 *vs* 16.47mm^2, p=0.7453). Interestingly, cavity area was dramatically reduced in rats receiving both stem cell transplantation *and* ChABC therapy compared to non-treated animals (2.72 *vs* 16.47mm^2, p=0.0309, Fig. 4.5, E). This decrease was also significant compared to animals treated with ChABC-only (p=0.084). Only the combinatorial therapy of ENSCs *and* ChABC resulted in a significant decrease in cavity area compared to non-treated animals. ANOVA analysis confirmed a significant difference between groups F(3, 16)=5.73, p=0.0074).

Lesion cavities tend to be surrounded by injured tissue, notable in both a disrupted organization and poor staining by eriochrome C. The significant reduction in cavity size by the combined ENSC + ChABC treatment prompted a further analysis of this injured tissue surrounding the cavity, to determine whether the reduced cavity area corresponded to an expanded area of injured tissue or an expanded area of preserved, uninjured tissue (Fig. 4.5, F). Quantification of the area of injured tissue area yielded results closely mirroring that of cavity area. ChABC treatment resulted in a decrease in the area of injured tissue (27.35 *vs* 32.72μm^2) that was not significant (p=0.728). ENSC transplantation resulted in a still greater decrease in the total area of injured tissue (21.83 *vs* 32.72μm^2), again this was not significant (p=0.213). The combined treatment resulted in the greatest

decrease in injured tissue area (17.43 *vs* 32.72μm^2). This decrease was significant compared to non-treated tissue (p=0.0408). ANOVA analysis confirmed a significant difference between groups (3, 16) = 3.622, p=0.0362).

Taken together, these results imply that the combined treatment results in an increase in the area of preserved tissue, by decreasing both the injury cavity and the area of injured tissue.

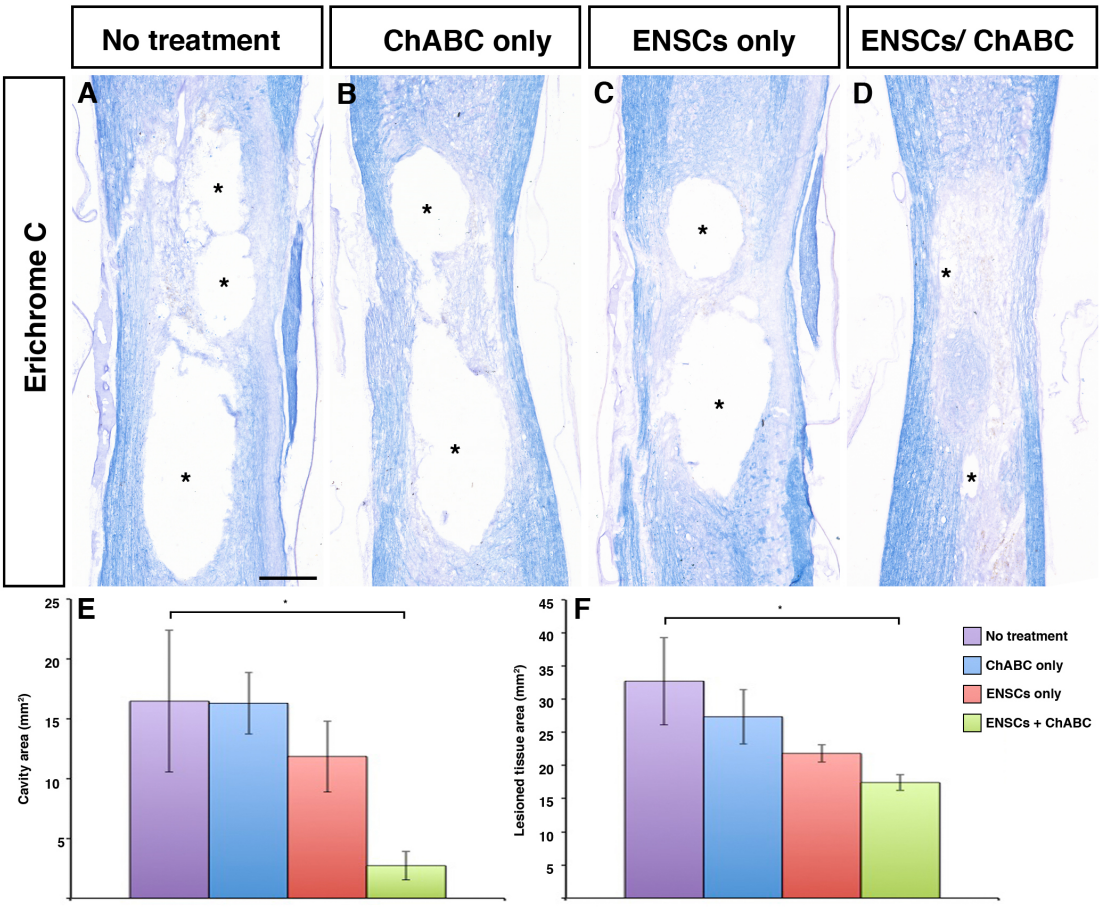

Figure 4.5: Quantification of spinal cord lesion histology using erichrome C staining.

Serial sections of harvested spinal cords harvested from animals sacrificed 16 weeks post-injury were stained for eriochrome C and the histology analysed. Area of either cavities or injured tissue was measured from the first section in which the lesion became apparent until it could no longer be detected. Results were then pooled to give total area. In the combined treatment group there was a significant decrease in cavity area ($p=0.0143$). Combinatorial treatment also resulted in a significant decrease in the area of injured tissue compared to both the ChABC-only treated group and non-treated group. Stem cell transplantation resulted in a decreased injured tissue area that was not significant, as did ChABC application. * indicates significance ($p=<0.05$). Scale bar: 500μm.

4.3.3 Survival and spread of transplanted ENSCs

The significant reduction in lesion cavity and injured tissue area detected in the ENSC + ChABC combined treatment group suggested an increased area of preserved tissue. This could be explained by either application of ChABC increasing survival/ spread of transplanted ENSCs (thereby reducing the lesion cavity by partially occupying it), or by the combined ENSC transplantation and ChABC application having a multiplicative effect on endogenous tissue survival. To determine any effect of ChABC on ENSC transplantation, spinal cord serial sections of rats receiving either ENSCs only, or ENSCs *and* ChABC were stained with an anti-GFP antibody and imaged. No significant difference between the ENSCs-only and ENSCs + ChABC was detected when cell spread was quantified, including spread along the A/P (2294.15 *vs* 2451.05, p=0.8573, Fig. 4.6, C), D/V (1325 *vs* 1025, p=0.3747, Fig. 4.6, D) and L/R (1074.55 *vs* 815.45, p=0.4335, Fig. 4.6, E) planes. The numbers of transplanted ENSCs detected in the two groups also was not significantly different (4077.75 *vs* 2724.5, p=0.167, Fig. 4.6, F). Similarly, quantification of the number of GFP+ pixels revealed no significant difference between rats receiving ENSCs-only and ENSCs + ChABC (542499.22 *vs* 543940.69, p=0.9956, data not shown). By all parameters quantified, co treatment with ChABC had no effect on transplanted ENSC survival or spread.

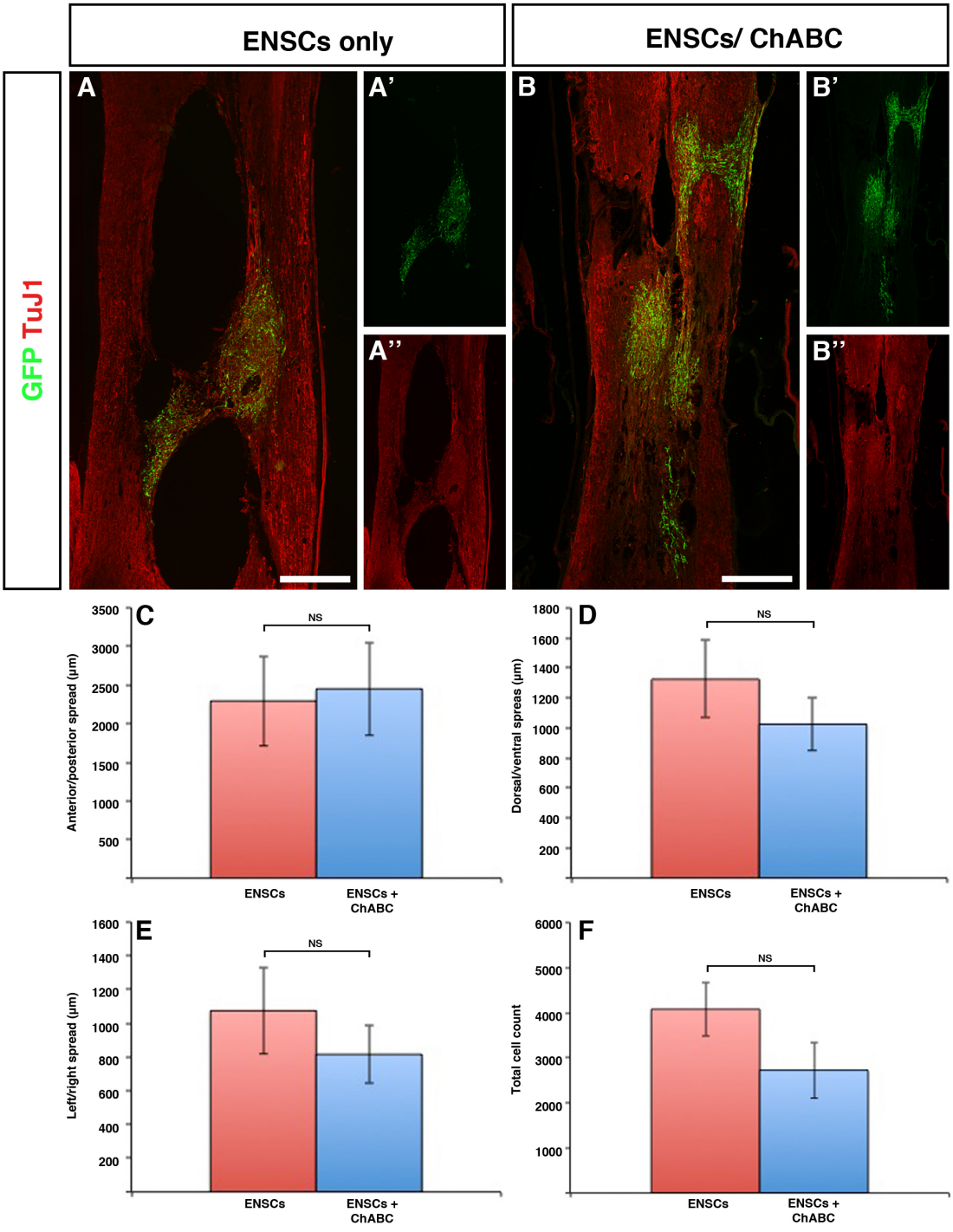
ENSCs only
ENSCs/ ChABC
GFP TuJ1
A
A'
A''
B
B'
B''
C
Anterior/posterior spread (µm)
3500
3000
2500
2000
1500
1000
500
NS
ENSCs
ENSCs + ChABC
D
Dorsal/ventral spreas (µm)
1800
1600
1400
1200
1000
800
600
400
200
NS
ENSCs
ENSCs + ChABC
E
Left/right spread (µm)
1600
1400
1200
1000
800
600
400
200
NS
ENSCs
ENSCs + ChABC
F
Total cell count
6000
5000
4000
3000
2000
1000
NS
ENSCs
ENSCs + ChABC

Figure 4.6: Quantification of transplanted ENSC spread and survival compared between animals treated with ENSCs only and animals treated with ENSCs + ChABC.

At the time of tissue harvest (16 weeks post-injury), numerous GFP+ cells were found within both the ENSC + ChABC-treated groups, and the ENSCs only-treated group (A, B). Serial spinal cord sections spanning the extent of GFP+ cell detection were analysed for cell spread across the anterior/posterior (C), dorsal/ventral (D), and left/right planes (E), as well as for cell survival (F). No significant differences were detected in any parameter between animals treated with ENSCs + ChABC or with ENSCs only. Scale bars: 500μm.

4.3.4 Transplantation of ENSCs results in a significant increase in the number of retrograde axons crossing the injury site

These results suggested that the significant reduction of cavity and injured tissue area seen in the combined treatment groups was due to increased preservation of the endogenous tissue rather than increased survival/ spread of transplanted ENSCs. To determine whether this tissue sparing resulted in an increased number of tracts bypassing the lesion, transplanted rats were injected with retro- and anterograde tracers, below and above the injury site, respectively. While BDA tracing proved weak and was restricted to the spinal cord periphery, usually not reaching the injury site, fluorogold injections resulted in extensive staining of neurons caudal to the injury site and substantial staining rostral of the lesion (Fig. 4.7). Morphology of the stained cells appeared variable, in terms of both size and number of processes. The majority of fluorogold+ cells was often located in the spinal cord centre, significantly anterior to the cavity. The number of fluorogold+ neurons rostral to the lesion was quantified in at least 3 sections at the injury epicentre and

compared across the non-treated, ENSC only, and ENSC + ChABC groups. No significant difference was observed between the ENSC + ChABC and ENSC only groups (89.95 *vs* 71.86, p=0.5158, Fig. 4.7, C). However, a significant difference was observed between the ENSC transplanted and non-treated groups (89.95 *vs* 34.3, p=0.0111, Fig. 4.7, C), suggesting that stem cell transplantation led to increased preservation or sprouting of retrograde tracts past the injury site.

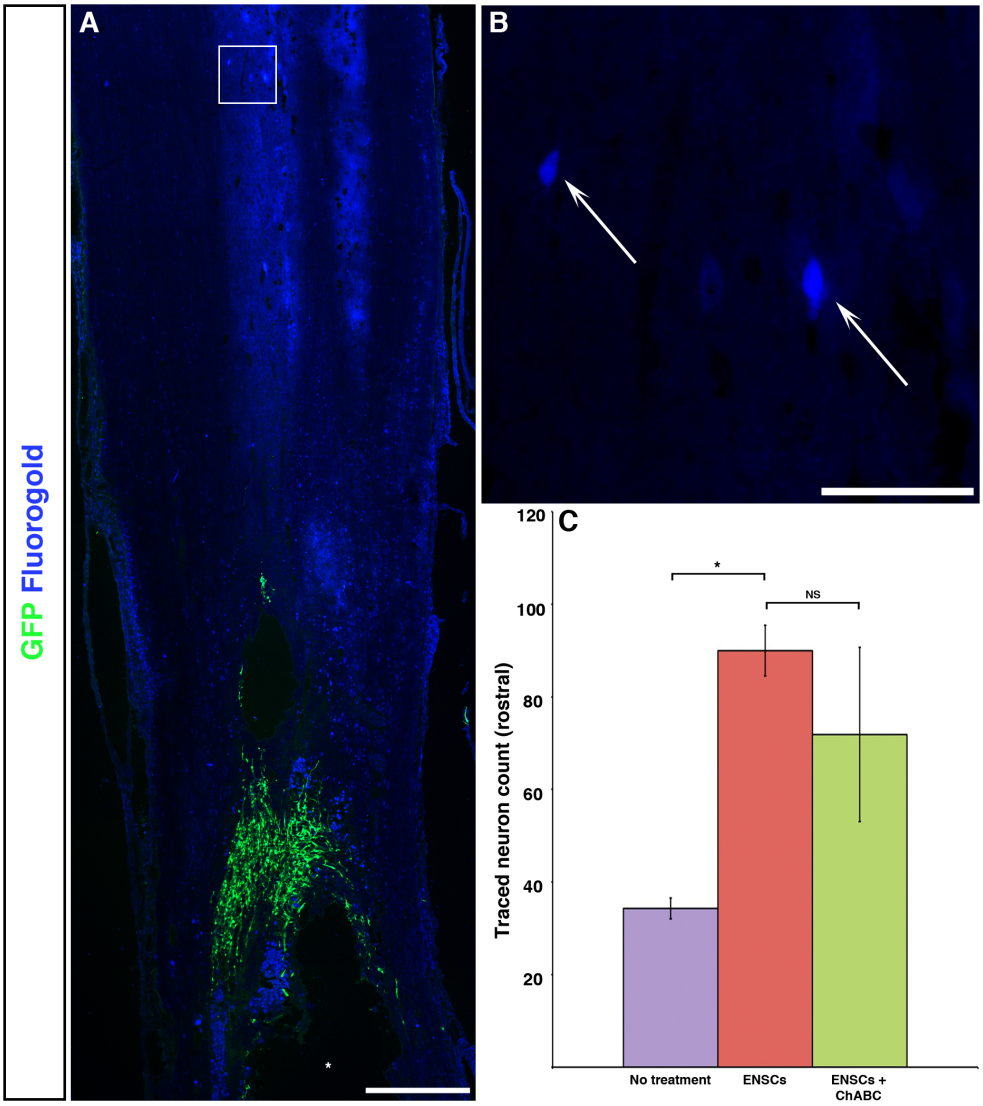

Figure 4.7: Quantification of fluorogold-labelled retrograde axons crossing the injury zone.

Fluorogold, a retrograde tracer, was injected 15 weeks post-injury (1 week prior to sacrifice of animals at week 16) caudal to the injury site. Following visualization of fluorogold, the number of fluorogold+ cells rostral to the injury cavity was quantified. This revealed a significant difference between transplanted and non-transplanted groups (p=0. 0111). A – tile scan of the injury epicentre and rostral spinal cord. Asterisk indicates injury cavity. Box indicates area magnified in B. Scale bars: A - 500μm, B - 100μm. No treatment – n=2, ENSCs only – n=2, ENSCs + ChABC – n=3. * indicates significance (p=<0.05).

4.3.5 Astrogliosis is reduced following ENSC only, ChABC only and ENSC + ChABC treatment

Reactive astrogliosis following SCI is a well-known phenomenon (Karimi-Abdolrezaee and Billakanti, 2012). Immunofluorescent detection of GFAP, a protein known to be upregulated following astrocytic activation (Ben Haim et al., 2015) was used to determine the extent of glial scarring in response to injury. GFAP+ staining was detected in SC sections taken from all groups (Fig. 4.8). GFAP+ staining was detected throughout the spinal cord, but was concentrated around the cavity perimeter and often the outermost white matter of the SC. Staining appeared more intense in the non-treated group, although it was not possible to quantify this in longitudinal sections.

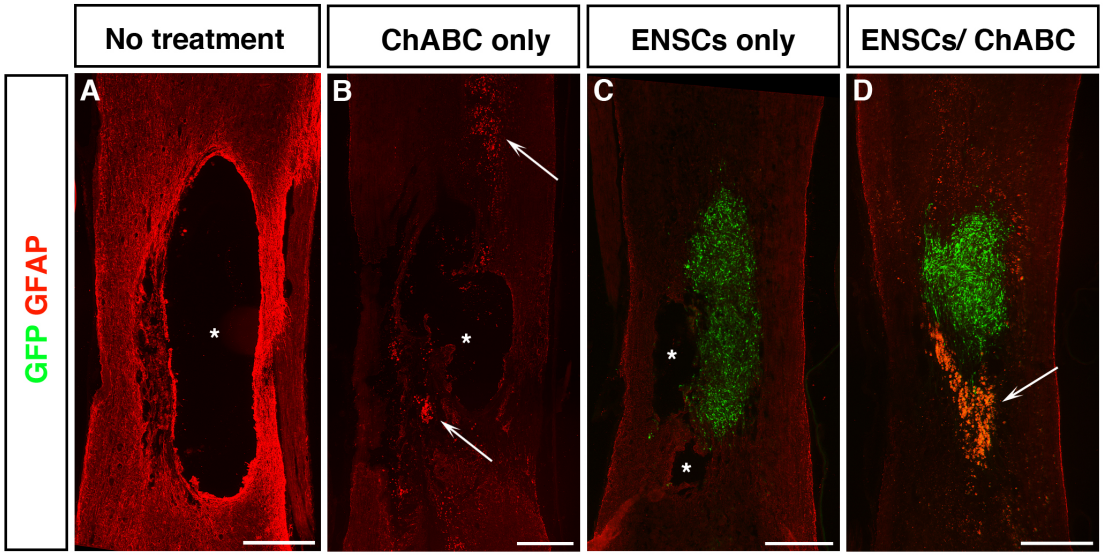

Figure 4.8: Comparison of glial activation between the four study groups using immunofluorescent detection of GFAP.

Longitudinal cryosections of spinal cord harvested 16 weeks post-injury were stained for the glial marker GFAP. Immunofluorescent detection of GFAP was strongest in the non-treatment group. In all groups, staining was particularly intense at the injury zone periphery. GFP+ staining of ENSCs was mostly distinct from GFAP+ astrocytic staining. Asterisks indicate lesion cavity, arrows indicate areas of autofluorescent material, possibly immune infiltration. Scale bars 500μm.

4.3.6 Assessment of transplanted cell spread to peripheral organs

Undesired migration and tumorigenesis of transplanted cells is a potential risk of stem cell-based treatments. No tumours were observed in any of the transplanted animals, and mortality was no different compared to non-transplanted animals over the course of the study. PCR was used to assess the spread of ENSC from the transplantation site in the injured spinal cord. Taking advantage of the GFP lentiviral label that transplanted cells had been transfected with, samples of peripheral organs were analysed for expression

of GFP. While all samples revealed expression of GAPDH, only positive control samples of transplanted SC revealed expression of GFP, with no GFP expression in all other organs examined (Fig. 4.9).

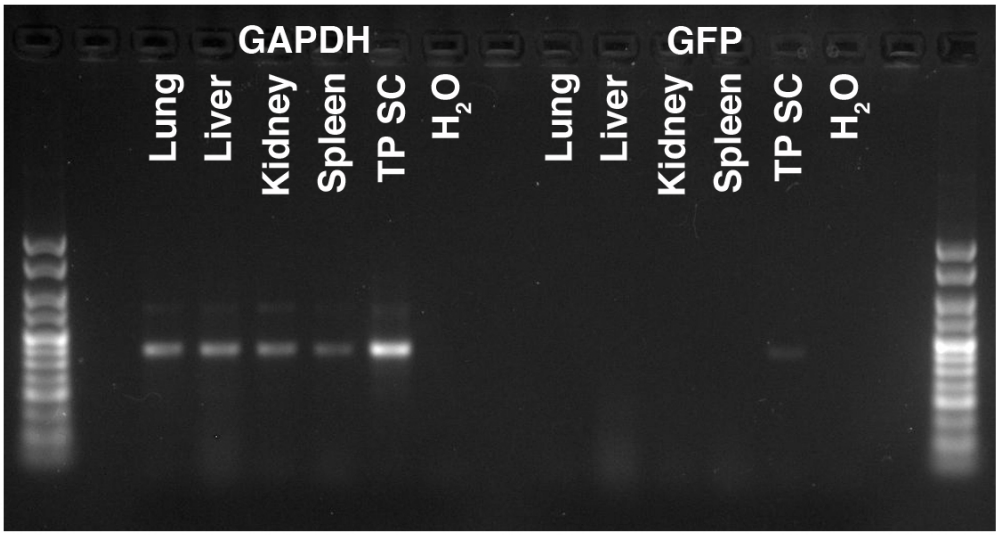

Figure 4.9: Assessment of spread of transplanted GFP+ cells to peripheral organs using PCR.

Samples of peripheral organs, including the lungs, liver, kidney and spleen were harvested from animals that had received transplantations of ENSCs into the SC. Genomic DNA was extracted and primers for GAPDH (control) and GFP were used to assess the presence of transplanted cells. Genomic DNA extracted from cryosections of transplanted rat SC confirmed to have GFP+ ENSCs, were used as a positive control (TP SC).

4.3.7 Behavioural analysis

The horizontal ladder test has been utilised previously as an assessment of motor function recovery following SCI. Beginning 1 week after injury, rats were assessed with this technique weekly up to 16 weeks (Fig. 4.10). All rats

gradually improved over the course of the study. The greatest improvement was observed in the first three weeks in all groups, with recovery apparently slowing after this point. There was no significant difference between motor improvement between treated and non-treated groups.

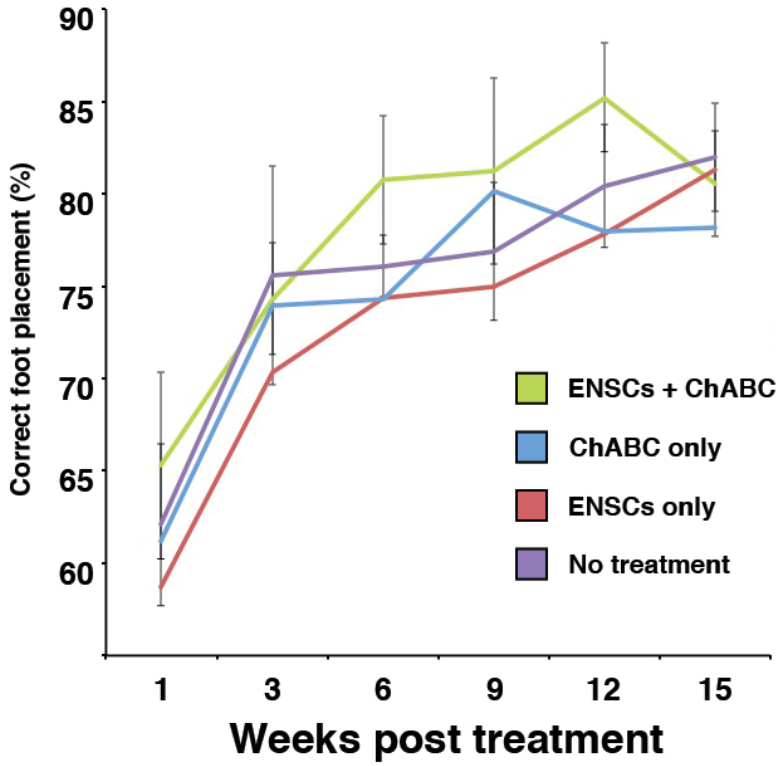

Figure 4.10: Horizontal ladder test measurement of motor improvements following SCI and treatment.

Starting one week after injury inducement and treatment, rats were assessed weekly for their ability to traverse the horizontal ladder. The results of three separate runs for each rat were pooled and the results expressed as percentage of correct foot placements. No significant differences in motor recovery were observed between groups (n=6 in each group).

4.4 Discussion

The complexity of the inhibitory microenvironment within the SCI zone provides a plethora of targets for therapies attempting to promote axonal regeneration. Treatments aimed at these have resulted in varying functional benefits, such as antibodies raised against myelin breakdown products (Liebscher et al., 2005). Arguably, one of the most successful approaches has centered on the accumulation of CSPGs within the glial scar. These are largely secreted by astrocytes within the scar and are thought to have a largely negative effect on axon re-growth. It has been exhaustively demonstrated that, collectively, CSPGs secreted in response to SCI have a negative impact on recovery (Fawcett, 2015; Siebert et al., 2014). Application of ChABC following CNS injury has previously been shown to increase plasticity and axonal sprouting, most likely through degradation of CSPG-rich perineuronal nets (Miao et al., 2014). Therefore, it was hypothesized that combinatorial application of ChABC with stem cell transplantation would increase either sprouting of transplanted cells, leading to enhanced bridge formation, or sprouting of endogenous cells to encourage them to cross the bridge formed by transplanted cells. Other authors have proposed similar combined treatments (Zhao and Fawcett, 2013).

4.4.1 Effects of single and combined treatment on lesion histology

Previous publications have observed a reduction in cavity size following various interventions (Hayashibe et al., 2016; Kim et al., 2017; Sarveazad et al., 2014), and it was posited that a similar reduction would follow treatment with ENSCs + ChABC. In line with this hypothesis, the greatest effects of

treatment on tissue histology were observed in the combinatorial group. Dual application of ENSC transplantation and ChABC resulted in significant reductions in both cavity area and affected tissue area. The effect of combined treatment was far greater than either ChABC virus or stem cell treatment alone. We observed no significant difference in the cavity area between ChABC only-treated and non-treated groups. Based on previous publications, it was expected that ChABC alone would significantly improve lesion histology (James et al., 2015). The lack of a significant reduction in cavity size may be due to variability in virus batches between the present and previous studies. We confirmed CSPG degradation in ChABC treated groups, and observed a non-significant reduction in the area of injured tissue in ChABC-treated animals compared to non-treated animals. This confirms that ChABC is present in the injected cords and has an effect, especially when used in combination with ENSC transplantation.

The results presented here, including the significant reduction in cavity and injured tissue area, and the paradoxical lack of significant difference in transplanted cell survival, as assessed by cell counting, GFP+ immunostaining threshold analysis of transplanted tissue, and transplanted cell spread analysis (along either A/P, D/V or L/R planes) implies that the positive effects of the combined treatment had instead acted on endogenous tissue. Previous stem cell transplantations have resulted in similar preservation of injured tissue. Zhou *et al* demonstrated a similar increase in fluorogold+ neurons rostral to the injury site following bone marrow mesenchymal stem cell transplantation into the injured SC. Further, they reported that this effect was increased following combinatorial application of a second therapy (propofol) (Zhou et al., 2015). Barbour *et al* reported significant increases in the number of fluorogold+ neurons rostral to the injury site following transplantation of either olfactory ensheathing glia or Schwann cells (Barbour

et al., 2013).

These results are in line with recent publications assessing combinatorial effects of ChABC application and stem cell transplantation as a therapeutic strategy for SCI. A study by Sarveasad *et al* tested the effects of combining human adipose-derived stem cell transplantation with ChABC on SCI pathology and motor recovery. While this study did not quantify lesion parameters such as cavity or injured tissue area, the authors observed a significant increase in the ratio of myelinated areas within the epicentre and the cavity area in the combined treatment group, exceeding the beneficial effects of either treatment alone (Sarveazad et al., 2017). The authors noted the tendency of mesenchymal-derived cells to reside within the cavity center as opposed to integrating into the surrounding tissue, implying that, as in the current study, application of ChABC led to beneficial effects through modulation of endogenous axons, as opposed to an action on the transplanted cells. This study did not, however, examine an effect of ChABC on transplanted cell survival. Another study by Fouad *et al* tested the effects of combinatorial ChABC and Schwann cell/OEG transplantation on regeneration of specific descending pathways (Fouad et al., 2005). Again, these authors observed the greatest effect in the combined group (although this study did not measure the effects of a ChABC-only group). Finally, a study by Suzuki *et al* demonstrated similar enhanced benefits of combined ChABC and NSC transplantation compared to either treatment alone, although this study used pre-treatment of the SC with ChABC to 'prime' the environment for later injection of stem cells (Suzuki et al., 2017).

The results of increased myelination following ChABC application observed by Sarveazad *et al* mirror the increased myelination in areas of ENSCs in the current study, as revealed by increased eriochrome c staining. It has previously been shown that invading peripheral Schwann cells can

remyelinate endogenous tracts following SCI (Beattie et al., 1997). As noted in the study by Sarveazad *et al*, CSPGs are able to inhibit migration, and it is probable that ChABC-mediated digestions allow greater ingress of Schwann cells to myelinate transplanted ENSCs.

4.4.2 Effect of single and combined treatment on astrocytic activation

Although not quantified, there appeared to be a reduction in immunointensity of GFAP staining in all treatment groups compared to the non-treated group. Considering astrocytes are the main source of the CSPGs found within the glial scar following SCI (Ohtake and Li, 2015; Yu et al., 2012) it may seem reasonable to assume that degradation of these CSPGs would lead to further activation. The decrease in activation observed could be explained by astrocytic response to C4S, the breakdown product of CSPGs. It is possible that detection of C4S by astrocytes leads to a negative feedback-activated down-regulation of CSPG production. Astrocytes contain an enzyme responsible for the degradation of C4S, arylsulfatase B (Zhang et al., 2014). It has been demonstrated in *in vitro* models of brain injury that astrocytes modulate the activity of arylsulfatase B in response to injury state, either causing and/ or in response to increasing levels of CSPGs/C4S. It is possible that astrocytes interpreted increased levels of C4S as increased levels of total CSPGs, leading to a reduction in astrocytic activity in ChABC-treated groups. Similar ChABC-mediated astrocytic reduction has been recorded by previous studies (Suzuki et al., 2017). Stem cell transplantation has also been shown previously to result in a decrease in astrocytic activation (Novikova et al., 2011), similar to the results observed in the current study.

4.4.3 Motor recovery following treatment

While there appeared to be a consistent trend towards increased motor recovery in the group receiving both ENSC transplantation and ChABC treatment, this was not statistically significant. Previous publications have noted significant improvements in motor recovery following either stem cell transplantation (McDonald et al., 1999), ChABC application (James et al., 2015) or a combined treatment (Sarveazad et al., 2017). Considering the combined treatment groups in the present study resulted in a significant reduction in cavity and injured tissue area, and that the number of fluorogold+ neurons traversing the injury site was likewise significantly increased in the ENSC-transplanted group compared to the non-treated group, the lack of any significant improvement in motor outcomes is perhaps surprising. However, it is worth noting that other studies have demonstrated significant improvements in one behavioral test (the open field BBB) but have then shown no significant improvement in the horizontal ladder test (Barbour et al., 2013). Thus, it is plausible that improvements in motor function were masked in the current study, and additional tests would have been required to discern them. Additionally, it has been previously shown that ChABC treatment resulted in significant motor improvements in severe, but not mild contusion injured rats (Caggiano et al., 2005). The authors did note a significant improvement in rats with a moderate contusion injury receiving ChABC, but the method of inducing injury varied between the current study and that by Caggiano *et al* (impact probe *VS* forceps compression of the spinal cord, respectively), making a direct comparison difficult. Thus it is possible that the injury induced in the current study was too mild to notice an effect. The timing of the treatment may also be key. James *et al* noticed a significant improvement in performance on the horizontal ladder following

treatment of moderately contused animals with ChABC, using the same methodology as the current study (James et al., 2015). Importantly, however, James *et al* administered ChABC *immediately* after inducing the injury, whereas in the current study, treatment was delayed by 3 days.

4.4.4 Cell spread

Transplanted ENSCs in the current study spread up to 4.2mm along the A/P axis. This spread is in line with that noted by McCann *et al* following transplantation of ENSCs into the colon (5.46±0.5mm^2) (McCann et al., 2017). However, post-acquisition mapping in the study by McCann *et al*, including deep-focussing through the entire tissue thickness, revealed that the longest spread in a single direction was actually 10.79mm. This disparity demonstrates the pitfalls of tissue sectioning. It is highly plausible that in the current study, axons extending from transplanted ENSCs traversed across individual sections, preventing them from contributing to the recorded spread. This could be more comprehensively assessed, by example, by tissue clearing to allow whole spinal cord samples to be analysed by immunofluorescence and subsequent microscopy.

The length of spread observed in the current study is also in line with transplantations of stem cells into the SC. Previous studies have observed spread of around 3-4mm following transplantations of dissociated human embryonic SC tissue (Wictorin and Bjorklund, 1992). Lepore and Fisher noted cell migration of up to 10mm following foetal stem cell transplantation, and up to 15mm following transplantation of co-cultured neuronal and glial progenitors (Lepore and Fischer, 2005). However, while the authors did not offer an average length of cell spread, they noted that it was highly variable, with some grafts showing only modest migration. Unfortunately, the vast

majority of stem cell transplantations do not quantify the spread of grated cells/cell-projections, instead mostly quantifying the re-growth of endogenous axons into the grafted cells and beyond (Kadoya et al., 2016). However, it is likely that both endogenous and graft-derived growth are important. Graft-derived axons extending into the endogenous tissue form an integrated bridge and may dilute the repulsive nature of the glial scar by crossing it. Further, extensive axonal growth of transplanted cells may encourage the same from endogenous cells, and provide paths leading both into and across the injury site.

Of note, there have been reports of greater spread. Lu *et al* observed extension of axons up to ~20mm (Lu et al., 2012). This impressive growth was seemingly brought about, at least in part, by the combinatorial application of fibrin scaffolds supplemented with a cocktail of growth factors including BDNF and GDNF among many others. It is possible that similar axonal growth could be induced from transplanted ENSCs if similarly supplemented. The authors also transplanted human NSCs into a rat model of SCI and found axons extending up to 9cm, an unprecedented level of growth (Lu et al., 2014). However, as was demonstrated by transplantations of human glia into mouse shiverer models, human glia have an ability to outcompete murine cells (Windrem et al., 2014). It is entirely possible, and indeed maybe even likely, that a similar effect is occurring following transplantation of human cells into the injured SC, considering the dramatic difference in these two studies which differed predominantly in the cell species transplanted. Indeed, it has even been shown that, in the shiverer model, human glial restricted progenitors are better able to rescue the phenotype and extend animal lifespan than their murine counterparts (Lyczek et al., 2017), further suggesting that the increased axonal growth of human axons compared to

murine may be due to species differences, and therefore unlikely to translate clinically.

4.4.5 Safety of ENSC transplantation

Tumorigenesis following stem cell transplantation is a recognized concern and a limiting factor of many stem cell applications. In a recent publication by Hirota *et al*, teratomas were observed in nearly a third of mice transplanted with iPSCs (Hirota et al., 2017). Indeed, careful assessment of teratoma formation following stem cell transplantation has been stated as vital for progression of a given stem cell therapy (Lukovic et al., 2014), and specific studies seeking to reduce tumerigenicity have been published (Fuhrmann et al., 2016). For this reason, the current study examined peripheral organs for obvious tumour formation, and finding none, conducted a more in depth PCR analysis of GFP expression (indicating the presence of GFP lentiviral-labelled ENSCs) in the lung, liver, kidney and spleen. GFP expression was only detected in transplanted SC tissue. These results are in line with previous results published by our group, in which long-term safety of ENSC transplantations into the ENS was assessed (Cooper et al., 2016). It should be noted that the current study has not ruled out a teratoma-driving effect of transplanted ENSCs on endogenous cells, although no tumours were noted in any animals under study. While an extended study of survival post-transplant will be necessary to provide further certainty of a lack of teratoma formation (as in the study by Cooper *et al* (up to 24 months)), these results provide initial evidence of the safety of ENSC transplantation into the SC.

4.4.6 Conclusions

Dual application of ENSCs and ChABC resulted in significant improvements in lesion histology surpassing those of either treatment alone. Combined application of ChABC and ENSCs resulted in similar survival and spread of transplanted cells compared to transplanted ENSCs-only. Further, both treatments increased the number of retrograde axons crossing the injury site, and reduced the amount of reactive astrogliosis occurring in the injury zone.

Chapter five: Overall Discussion and Future Directions

5.1.1 Summary

Work in this thesis clearly demonstrates the potential of ENSCs for SCI therapy. *In vitro* studies using the chick demonstrated the potential of ENSCs to produce and replenish the neuronal subtypes lost to SCI, and provided proof of principle data demonstrating the ability of ENSCs to integrate with SC-derived cells. *In ovo* work demonstrated the ability of ENSCs to survive following transplantation into the embryonic chick spinal cord. While these results were encouraging, several features of the embryonic chicken necessitated a progression to an adult animal model of SCI. Perhaps most importantly, the embryonic chick is known to have a limited immune system at the time points under examination in the current study, with immature T cells incapable of an immune response present within the thymus, and none in the spleen by E13.5 (Lowenthal et al., 1994; Seto, 1981). Indeed, even in the initial weeks post-hatching, the immune system of chickens is notably poor and many succumb to infection (Wallach et al., 1992). Additionally, astrogliosis and myelin degradation in response to SCI are not observed until after E13 (Ferretti et al., 2003), prompting additional studies in a model organism that better recapitulates the environment seen in adult SCI. The rat was chosen as the most suitable adult model, and a pilot study conducted to determine the optimal time post-injury for cell transplantation. This demonstrated the ability of ENSCs to survive in the SC injury zone for up to 2 weeks, and prompted a larger scale rat study with a more severe SCI, for an extended period of time. This extended study assessed the benefit of a combinatorial therapy consisting of dual application of transplanted ENSCs and the bacterial enzyme ChABC, a modifier of the inhibitory microenvironment. Treatment outcomes examined in this study focused mostly on tissue histology, including both cavity and injured tissue area.

Further, this part of the thesis showed that ENSC transplantation resulted in a significant increase in the number of retrograde axons crossing the injury site, a potential increase in myelination within the injury zone and a reduction in astrogliosis. Overall, this work provides data supporting the idea that enteric nervous system stem cells, derived from the gastrointestinal tract, could, in the future, be used as therapy for SCI.

5.1.2 Discussion

5.1.2.1 Safety of ENSC transplantation

A key concern following any stem cell treatment is safety (Fan et al., 2017). This includes cell migration outside of the transplantation area, differentiation towards undesirable cell fates, and uncontrolled proliferation leading to tumour formation. These issues must be addressed before progression of stem cell transplantations towards clinical trials.

Results of chapter 2 demonstrated a tendency of transplanted ENSCs to localize in TuJ1+ neuronal tissue. While some spread into the PNS through dorsal root ganglia was observed in the earlier time points examined (E5.5, and to a lesser extent E7.5), by later stages of transplantation this spread appeared to be much reduced. At all stages, the vast majority of GFP+ ENSCs remained in the SC. Additionally, there are several reasons why the spread observed in early embryonic stages may not occur following ENSC transplantation into an adult model of SCI. Firstly, transplantation into the chick neural tube involved complete removal of a segment of the neural tube and grafting of a neurosphere into the ablation. Transplanted cells were thus in contact with the developing dorsal root ganglia, which would not be the

case following injection of ENSCs into the injury cavity in an adult model of SCI. The dorsal root ganglia and peripheral nervous system are also of neural crest origin (Kasemeier-Kulesa et al., 2005; Newbern, 2015; Teillet et al., 1987). Transplanted ENSCs may therefore have been attracted to cues secreted by these developing structures (Belmadani et al., 2005; Britsch et al., 1998; Newbern, 2015), and may have even contributed to the development of these structures. An adult model, in which PNS development is long since completed, would not provide such cues, possibly meaning ENSCs would be less likely to spread in such a manner. This conclusion was supported by transplantations into the rat SC, in which ENSCs were observed mostly within the centre of the cord surrounding the injury zone.

In terms of safety, cell fate may be as important as location. In all *in vivo* studies detailed in this thesis, extensive neuronal differentiation of ENSCs was observed post-transplantation, as assessed by TuJ1 immunostaining. This was supported by the *in vitro* chick studies described in chapter 2, in which qRT-PCR revealed expression of a panel of neuronal subtype genes within ENSC cultures. These results are in line with previous work involving ENSC transplantation into the ENS, in which neuronal differentiation of ENSCs appeared to be the default pathway (Cooper et al., 2017; McCann et al., 2017; Natarajan et al., 2014).

This cell fate characterization could be extended further to determine whether there are any other cells contained within the ENSC-derived population post-transplantation. While this would be unexpected following FACS isolation based on neural crest lineage, such an analysis would be important to address safety concerns. For example, the presence of mesenchymal cells could be assessed. Several reports have suggested differentiation capability of ENCCs towards a mesenchymal lineage. A study by Li *et al* demonstrated the potential of hiPSC-derived neural crest stem cell

to differentiate into CD44, CD73 and CD90-expressing mesenchymal cells (Li et al., 2016b). However, driving these cells towards an induced pluripotent state is substantially different from the usual fate of these cells, and mesenchymal differentiation may be an artefact of this process. Another report by Lee *et al* showed mesenchymal differentiation of neural crest cells, but these were derived from embryonic stem cells and driven to their neural crest fate *in vitro*, again likely reflecting dramatic differences in differentiation to that which occurs *in vivo* (Lee et al., 2007). A recent publication by Kumar *et al* noted that, while driving hPSCs to a mesenchymal lineage, cells passed through a stage where the surface antigen p75 was expressed (Kumar et al., 2017). p75 is the same marker used in the current study to isolate neural crest-derived cells from surrounding rat tissue using FACS (chapters 3 and 4). Again, however, driving cells down this lineage is likely very different to that which occurs *in vivo*. Indeed, it has been noted that the various unnatural environments of *in vitro* culture can cause cells to behave in ways that may not occur *in vivo* (Edmondson et al., 2014). All *in vitro* work therefore, while very informative, should be interpreted with caution. Additionally, mesenchymal differentiation of ENSCs has not previously been noted following transplantation into the ENS (Cooper et al., 2016; McCann et al., 2017; Metzger et al., 2009b), making it unlikely that any such differentiation would be observed in the current study. Nevertheless, an assessment of any ENSC mesenchymal differentiation potential should be carefully assessed in future studies. Ideally, this would be complemented with an *in vivo* corroboration following transplantation.

Finally, tumour formation was not observed in transplanted rats in the pilot or extended study described within this thesis, and nor was any difference in mortality observed following transplantation. PCR analysis revealed no GFP expression in the peripheral organs under examination

(lung, liver, kidney, spleen) in three transplanted rats, indicating transplanted ENSCs had not spread to these sites. Therefore, none of the observations within this thesis warrant safety concerns for ENSC transplantation.

5.1.2.2 Possible dual roles of CSPGs

Modulation of specific aspects of the glial scar has shown great promise towards enhancing SCI recovery. In particular, studies in which CSPGs were depleted within the injury zone have led to histopathological and functional improvements following SCI (Bartus et al., 2014; James et al., 2015). Developmental studies have supported these findings, demonstrating that degradation of CSPGs, via ChABC, leads to an extension of the 'plastic' window of neurodevelopment (Pizzorusso et al., 2002). The histological improvements in tissue preservation observed in this study avidly support such research and encourage further work into combining stem cell transplants with modifiers of the inhibitory microenvironment. Again, however, this may not be the whole picture. Early in development, CSPGs have been shown to have critical roles in axon guidance (Silver and Silver, 2014), and it is plausible that CSPGs within the injury zone may, at least in part, be an attempt to fulfill a similar role. Further, a study by Rolls *et al* demonstrated that removing CSPGs entirely has a detrimental effect on motor recovery, while, similar to findings concerning the glial scar, removing it only in the chronic phase of SCI has a beneficial effect (Rolls et al., 2008). Further, the authors noted a role for CSPGs in microglial/ macrophage activation, and even a role in encouraging the former towards a pro-neuronal survival phenotype. The authors had previously shown that one of the breakdown products of CSPG degradation, 6-sulfated disaccharides, has neuroprotective effects (Rolls et al., 2004), and demonstrated its ability to

improve functional outcomes following injection into the injured SC. Taking into account the extensive studies detailing the advantages of removing CSPGs from the injury site, this implies that any positive effect provided by CSPGs at the early stages of SCI is largely overwhelmed by other, negative effects at the later stages. However, future refinement of CSPG ablations aimed at depleting CSPGs in a specific time frame could result in a greater improvement in SCI prognosis, especially if designed in conjunction with deliberate alternatives for neuroprotective macrophage/ microglial activation.

5.1.2.3 Motor improvements following ENSC + ChABC treatment

The ultimate goal of any treatment for SCI, especially as stated by patients, is the recovery of motor function (Haas et al., 2016). An increase in motor function is invariably linked to improvements in tissue histology following SCI (Barbour et al., 2013; Karimi-Abdolrezaee et al., 2010; Xiong et al., 2017). The studies in chapter 4 demonstrated significant improvements in injured SC tissue histology following the combined ENSC + ChABC, including reduction in cavity/injured tissue area and increased retrograde axons crossing the injury site. Paradoxically, however, while there was a trend towards increased recovery in the combined treatment group, the significant histological improvements did not translate into a significant functional improvement. There are a number of reasons why this could have been the case, including a failure to find an improvement that nevertheless existed. Additionally, extensive further work could be performed to increase the effectiveness of the ENSC + ChABC combinatorial treatment.

A recent publication tested a similar stem cell/ ChABC combined treatment to that assessed within this thesis (Suzuki et al., 2017). Here, the authors noted a significant improvement in motor recovery following

combinatorial treatment with iPSC-derived NSCs, in contrast to the work presented in this thesis. As stated in the previous chapter, this discrepancy may well be due to the particular types of test utilised (forelimb grip strength and catwalk analysis in the study by Suzuki *et al*, and the horizontal ladder test in this thesis), and undoubtedly demonstrates the need for more behavioural examination of treated rats in any follow up studies of ENSC + ChABC treatment. The method of injury inducement was also different (cervical clip-contusion method by Suzuki *at al*, thoracic impact probe-induced contusion in this thesis), as was the window of stem cell transplantation (chronic stages of SCI by Suzuki *et al*, compared with acute phase transplantation in this thesis).

However, differences in the treatment application may also be worth consideration. Suzuki *et al* treated injured SC with ChABC delivered via an osmotic pump for 1 week prior to stem cell transplantation, as opposed to co-injection as in this thesis. A similar approach using ENSCs and the lentivirus-containing ChABC could be assessed in a future study. As discussed in chapter 3, injection of ENSCs at an earlier stage was ruled out because the glial scar appeared to prevent transplanted cells from localising to the injury zone. However, pre-treatment with ChABC a week or so before transplantation may alleviate this concern, and as in the study by Suzuki *et al*, thereby elicit improved motor activity due to the primed environment. Additionally, the detection of CSPG breakdown products, used to determine ChABC activity in this thesis, is not easily quantifiable, and while unlikely, it is possible that co-transplantation had an effect on enzyme activity. Measuring the activity of the enzyme itself would provide a more reproducible and accurate assessment of CSPG degradation over time, for which effective methods exist (Lee et al., 2010). An assessment of the activity across the different groups at timed intervals post-injection would more accurately

determine any difference in enzyme activity between the groups, allowing any treatment outcomes to be more robustly examined. This would provide an important check to rule out differences in enzyme activity between the ENSC + ChABC and ChABC-only groups.

5.1.2.4 Reactive astrogliosis in spinal cord injury

SCI results in an incredibly complex cascade of events leading to neurodegeneration (Kjell and Olson, 2016; Vismara et al., 2017). Although extensive research has focused on the mechanisms and molecular components responsible for cell death and the inhibitory microenvironment of the injury zone (Iaci et al., 2007; Plemel et al., 2014; Zhang et al., 2012), our knowledge of these events is far from complete. Indeed, even the effects of well-documented processes are sometimes unclear. For example, the role of the glial scar, long thought to be a potent barrier to regeneration, is now in question. Astrocytic activation in response to injury of the central nervous system is a well known phenomenon (Reier and Houle, 1988). Indeed, within the SC, astrocytic activation and proliferation is a pathological hallmark of the injury process (Yuan and He, 2013), and its reduction following treatment is often used as an indicator of success (Hara et al., 2017; Novikova et al., 2011; Xiong et al., 2017). Concordantly, in chapter 4, qualitative analysis showed a reduction in astrogliosis surrounding the injury zone following application of ENSCs, ChABC, or both. Broadly speaking, previous interpretations of a beneficial outcome of such a reduction are likely correct. However, extensive literature has demonstrated that this may not be the whole story, and that there may be functional roles for astrocytes in improving injury outcomes (Liu et al., 2014; Lukovic et al., 2015; Renault-Mihara et al., 2008).

Indeed, negligible or negative effects on motor recovery have been observed following resection of the glial scar, with the outcome dependent on the type of injury inflicted (Rasouli et al., 2009). A reconciliation for these opposing results has been offered by Rolls, Schechter and Schwartz (Rolls et al., 2009) who proposed that these differing effects of the glial scar may be due to timing, whereby early in the injury process the glial scar is beneficial, but later becomes detrimental to recovery (similar to the proposed mechanism of CSPGs, see above). For this reason, it would be important for future ENSC transplantations to determine an optimal window in terms of glial scar progression. The pilot study detailed in chapter 3 elucidated that early transplantation was advantageous for ENSC localization to the injury zone. It is arguable that the primary function of the glial scar is in restricting expansion of the lesion (Li et al., 2008). Thus, in the acute phase of injury, transplanting ENSCs before maturation of the scar may have the obvious benefit of allowing ENSCs to integrate before astrocytes have had sufficient time to increase the concentration of axon growth-retarding CSPGs. However, doing so may prevent the glial scar from achieving its initial goal of limiting lesion spread. A more effective strategy may, therefore, be to allow the glial scar to form and exert its beneficial effects, before injecting ChABC as a 'primer' to reduce the repellant nature of the scar and, after a suitable time period, transplant the ENSCs. This would allow any beneficial effects of the glial scar on the acute phase to be combined with the growth encouraging properties of the transplanted ENSCs.

5.1.2.5 Effect of SCI on the ENS

The ENS provides an easily accessible source of NSCs for autologous treatment, as an alternative to the stem cell niches of the brain and spinal

cord (Decimo et al., 2012). While other NSC sources, such as OECs, are relatively accessible compared to extraction from the brain or spinal cord, their harvesting would still involve significant surgical risk due to close proximity to the brain (Barnett and Riddell, 2004; Tabakow et al., 2014). Additional advantages detailed in chapter 1 include the ability of the enteric and central nervous systems to communicate via several nerve tracts, utilising a common neurotransmitter pool. However, these extensive connections mean that significant damage to one system may affect the other. For example, it has been suggested that neurodegenerative diseases such as Parkinson's disease (PD) may originate in the ENS and thence travel rostrally to the brain (Pan-Montojo et al., 2010). Similarly, previous work has indicated that SCI can have an effect upon enteric physiology (Awad, 2011; den Braber-Ymker et al., 2017; Guizar-Sahagun et al., 1996), with constipation in particular being linked to a patient's quality of life (Adriaansen et al., 2016).

It is unclear what effect, if any, SCI would have on ENSCs. Evidence suggests that the gastric dysmotility arising from SCI is due to the effect of a disrupted signalling from the central to the enteric nervous system (Holmes, 2012; Hou and Rabchevsky, 2014), implying that such dysmotility occurs at a system level rather than a cellular level. Therefore it is plausible that any effects of SCI on GI motility via enteric dysfunction would potentially have no impact on harvest, culture and therapeutic use of ENSCs. Nevertheless, an important step in providing evidence that ENSCs could serve as a stem cell source for SCI would be to demonstrate that biopsy-derived ENSCs isolated from animals with SCI would not be adversely different from ENSCs isolated from animals without SCI. This would require extensive *in vitro* comparison including RNAseq, qRT-PCR and immunofluorescent comparisons of cell fate potential between the two groups, as well as an evaluation of the behaviour of such cells. In particular, differences in the proliferative, migratory and

axonal extension capabilities should be compared between the two groups. This could be further tested by assessing the therapeutic benefits of autologous transplantation of ENSCs harvested *after* inducing SCI, and comparing this with therapeutic benefits of autologous transplantation of ENSCs harvested *before* inducing SCI.

5.1.3 Limitations and future work

There are several ways in which the scope of the current study could be expanded, and which will serve as useful guidelines for future work on ENSC transplantation. Firstly, extended n numbers of each study group would allow for more extended histological analysis. The studies described in chapter 4 were limited to sagittal sectioning. However, transverse sectioning arguably allows for more accurate quantifications of grey and white matter loss, as these can be expressed relative to the area of the whole cord. Greater numbers within each group would also allow for further quantitative assessments. Serial sections obtained in the current study were filled five slides at a time, with five sections on each slide. This allowed each slide to show a range of section depths, but limited immunofluorescent or other quantitative assessments to five tests per animal. Greater n numbers would allow for further quantitative assessments, for example to provide comparisons between C4S and GFAP staining intensity (especially if these samples had been sectioned transversely).

5.1.3.1 Assessments of motor recovery

The failure of any one test to accurately gauge all aspects of functional motor improvement has been noted (Onifer et al., 2007). Indeed, it has been reported that significant increases in functional improvement may have gone unreported due to variability between the specific motor skills assessed by a given test (Fouad et al., 2013). Therefore, as mentioned above, the use of the horizontal ladder for improved motor outcome could be supplemented with additional tests. For example, tests exist to ascertain recovery of specific tracts. Corticospinal/ rubrospinal tract recovery can be assessed by reaching and grasping tests (Kanagal and Muir, 2007; Morris et al., 2011). Qualitative analyses such as the BBB score can analyse compensatory mechanisms that may otherwise mask underlying motor recovery, or a lack thereof (Fouad et al., 2013). For example, rats with hind limb deficits often swim more forcefully with their forelimbs to compensate (Smith et al., 2006). Such rats thus appear to perform well following injury, and can occasionally outperform non-injured animals, but true motor improvement involving recovery of the pre-injured tract affected, has not occurred. Therefore, a true assessment of motor recovery following SCI and/ or treatment would involve both specific, quantitative tasks as well as a qualitative task to unmask any compensatory mechanisms, and this will form an important core component of future work into ENSC transplantation into the injured SC.

5.1.3.2 Additional measures of functional improvements

Behavioural tests are not the only method of assessing functional improvement following SCI. Electrophysiology has been successfully used to evaluate conduction across the lesion site (Hains et al., 2004). Further,

increased conduction was shown to correlate highly with improved performance on the horizontal ladder test, but not with functional gains detected by BBB assessment (James et al., 2011). This again underlies the importance of utilizing multiple behavioural tasks, and supports the use of electrophysiology as an accurate alternative technique for measuring functional improvements. While James *et al* noted a correlation between motor improvement in the horizontal ladder and axonal conduction across the injury site, it is possible that electrophysiological analysis of animals in the current study would have shown increased axonal conduction despite no significant improvement on the horizontal ladder test. Significantly more retrograde axons crossed the injury site in ENSC transplanted vs non-transplanted animals in the current study, suggesting that increased axonal conduction is likely. Other physiological assessments, including live calcium imaging, are also alternatives. This has been successfully demonstrated in both neurons (Sekiguchi et al., 2016) and astrocytes (Cirillo et al., 2012), the latter of which may be particularly relevant in the context of ChABC application.

5.1.3.3 Post-transplantation characterization of ENSCs and their mode of action

The chick studies described in chapter two involved characterization of the ENSC neurospheres. qRT-PCR revealed the presence of a range of neuronal subtype genes within ENSC neurospheres, indicating the presence of nitrergic, serotonergic, glutaminergic, acetylcholinergic and GABAergic neurons. Although not performed, it is highly likely that qRT-PCR analysis of rat-derived ENSC neurospheres would show no major differences, as studies of mouse neurospheres from our lab had similar neuronal profiles to that

observed in the chick studies of this thesis (Binder et al., 2015; Cooper et al., 2016). However, such a characterization would prove vital if ENSC transplantations were to progress to a clinical setting. Indeed, the characterization would need to be further refined, including the use of immunolabelling to confirm the presence of specific neuronal subtypes *in vitro*. It would be of additional benefit to determine the differentiation potential of ENSCs post-transplantation. Indeed, any such results should be quantified (either by immunostaining, or qRT-PCR analysis of ENSCs FACS isolated from dissociated tissue following transplantation) and these results compared to pre-transplantation results. Any differences in expression of particular neuronal subtypes pre- and post-transplantation would be highly informative. If a particular subtype decreases, this could be due either to an increased vulnerability to the inhibitory SCI microenvironment relative to other subtypes, or because other subtypes were more relevant to SCI recovery and 'selected for' via signals from endogenous, surviving cells. Either result would inform future studies. Additional support, such as immune cell suppressors, could be applied if the decrease is due to higher vulnerability. Selection via endogenous-driven signalling could be assessed by determining the extent of functional synapse formation between endogenous and transplanted cells. This could be achieved via immunolabelling for synaptic proteins, such as synaptophysin and electron microscopy analysis of such synapses to determine presence of specific synaptic elements, such as distinguishing the pre- and post synapses (Navlakha et al., 2013). Endogenous secreted differentiation factors could be elucidated by removing transplanted ENSCs (via FACS) and using a proteomic approach, such as mass spectrometry analysis, to assess dissociated endogenous cells (Devaux et al., 2016; Henningsen et al., 2010; Yan et al., 2010).

This approach would also be particularly important for elucidating the specific roles of ENSCs following transplantation into the injured SC. Considering that the greatest reduction in cavity area and injured tissue occurred in the combination ENSC + ChABC group, but there was no significant difference in transplanted cell survival or spread between the combination and ENSC-only groups, this implies that the major role of ENSCs, together with ChABC, was in preserving endogenous tissue. However, it remains to be seen whether this occurred through a paracrine or autocrine method. Previous stem cell transplantations have often demonstrated the potential of transplanted stem cells to secrete neurotrophic factors (Dasari et al., 2014; Lu et al., 2003) and even revealed specific mechanisms by which paracrine-driven preservation of endogenous tissue may occur (Yan et al., 2004). While the results described within this study provide evidence that ENSCs transplanted into the injured SC fulfil a similar paracrine-driven effect on endogenous tissue, future work will need to elucidate specific mechanisms by which this effect occurs.

5.1.3.4 Potential for co-injection of ENSCs + ChABC

Separate transplantation of ENSCs and ChABC injection within the current study likely introduced additional tissue damage through needle penetration. While such damage is thought to have little effect on recovery *per se*, a reduction in the number of injections would reduce the surgical risk of further paralysis if this treatment were to reach clinical trials. If a 'priming' injection of ChABC before ENSC transplantation was not used, one method to reduce the invasiveness of this procedure would be to combine the ENSC transplantation and ChABC injections. Application of the ChABC-containing lentivirus to the ENSCs *in vitro* would remove the need for further ChABC

injections. The success of lentiviral transduction would need to be assessed prior to transplantation. This could be achieved by testing the ability of transduced ENSCs to extend axons along CSPG coated dishes (Snow and Letourneau, 1992), and potentially by an assessment of the enzyme activity as mentioned above. Providing the ability of ENSCs to produce the ChABC enzyme could be verified, these could then be transplanted into an injured spinal cord to provide both the stem cell and ChABC components of the combinatorial therapy. The efficacy of this single, combined application would need to be compared to the outcomes of the combinatorial therapy applied separately.

5.1.3.5 Use of scaffolds for other categories of SCI

This study assessed the potential of ENSCs to preserve and rescue endogenous tissue following a contusion SCI. As detailed in the previous chapters, this injury model was selected as being the most relevant to human SCI. However, contusions are not the only injury type, and each presents with unique challenges (Cheriyan et al., 2014; Kandziora et al., 2016; Sharif-Alhoseini et al., 2017; Yue et al., 2016). It is important that prospective stem cell-based therapies are able to adapt to these varying needs if progression to a clinical setting, and to the broad range of patients with SCI, is to succeed. While the ENSC + ChABC combinatorial treatment described within this thesis holds substantial promise, additional modifiers may be necessary to adapt this treatment to each injury type. With transection injuries, for example, in which the spinal cord is partially or completely severed, a significant gap can often exist between the anterior and posterior spinal cord tissue. In such instances, a physical bridge may be required, which could then be populated with ENSCs to encourage endogenous regeneration across the

injury site. Scaffolds of varying composition have been tested for their therapeutic benefit, and many have shown significant promise towards SCI recovery. These have included both biological scaffolds, such as peripheral nerve grafts (Cote et al., 2011) and synthetic polymer-based scaffolds (Teng et al., 2002). Particular promise has been demonstrated in a range of hydrogel scaffolds (Agbay et al., 2016). Many hydrogel scaffolds have the advantage of being injectable, ensuring complete filling of the cavity with minimal invasiveness (Slaughter et al., 2009). Of pertinence to the current study, stem cells can be diluted in hydrogel scaffolds as a cell suspension, allowing simultaneous injection and an even dispersion of stem cells throughout the scaffold. This technique has been shown to improve survival rates of stem cells following transplantation into a CNS environment (Ballios et al., 2015). This technique may therefore be applicable for enhancing the survival of the ENSCs described within this thesis in injury models where SC transection or a particularly severe contusion has resulted in formation of cavities too large to be filled by stem cell transplantation alone, and would be a useful additional modification for ENSC transplantation therapy in future studies.

5.1.3.6 Progression to larger animals to demonstrate autologous transplantation

Previous stem cell studies proposing autologous transplantation have often turned towards the use of iPSCs (Khazaei et al., 2016). But, as highlighted in chapter 1, despite their increasing promise, iPSC generation still holds significant barriers to success, including tumerogenicity concern regarding genetic instability (Kuroda et al., 2013; Lund et al., 2012; Yoshihara et al., 2017). This thesis demonstrates the potential of ENSCs to serve as an

alternative autologous stem cell source for SCI repair. ENSCs have previously been isolated and expanded *in vitro* from human gut biopsies (Binder et al., 2015) and separately, this thesis has shown that ENSCs can be transplanted into the CNS environment of the SC. This work is supported by a recent publication describing success of ENSC transplantation into the brain (Belkind-Gerson et al., 2016), and shows the potential of stem cells isolated from the enteric nervous system to survive in other nervous systems, expanding the range of neural disorders to which this stem cell source could be applied. However, as yet ENSC studies have been limited to allogenic transplant, involving either extraction from one animal and transplantation into another (as described in this thesis and the work by Belkind-Gerson *et al*, among others), or extraction from human tissue and transplantation into animal gut (Cooper et al., 2017). These studies provide important proof of principle data suggesting autologous transplantation could be successful.

However, to fully demonstrate this and determine the functional benefit of such transplantation, progression to a larger model animal would be vital. Use of a model organism such as the pig, would allow stem cell isolation from gut tissue biopsies and subsequent expansion *in vitro,* followed by transplantation into the same animal with SCI (Fig. 5.1) Such autologous transplantation is technically challenging in small animal models such as rodents. Given the underlying similarities with human biology and their ready availability, pigs are becoming increasingly popular as a large animal model for study (Walters et al., 2017). Porcine models of SCI have been developed to measure a variety of SCI outcomes (Jones et al., 2012; Lee et al., 2013; Schomberg et al., 2017), and it has even been suggested that these may offer significant improvements over existing rodent models due to a greater similarity between the porcine and human spinal cord (Leonard et al., 2017). The pig is also currently being examined for utilization

towards ENSC-based cell therapies for enteric neuropathies (Burns et al., 2016). Collectively, this demonstrates the feasibility and promise of progressing ENSC transplantation to a large animal model of SCI such as the pig.

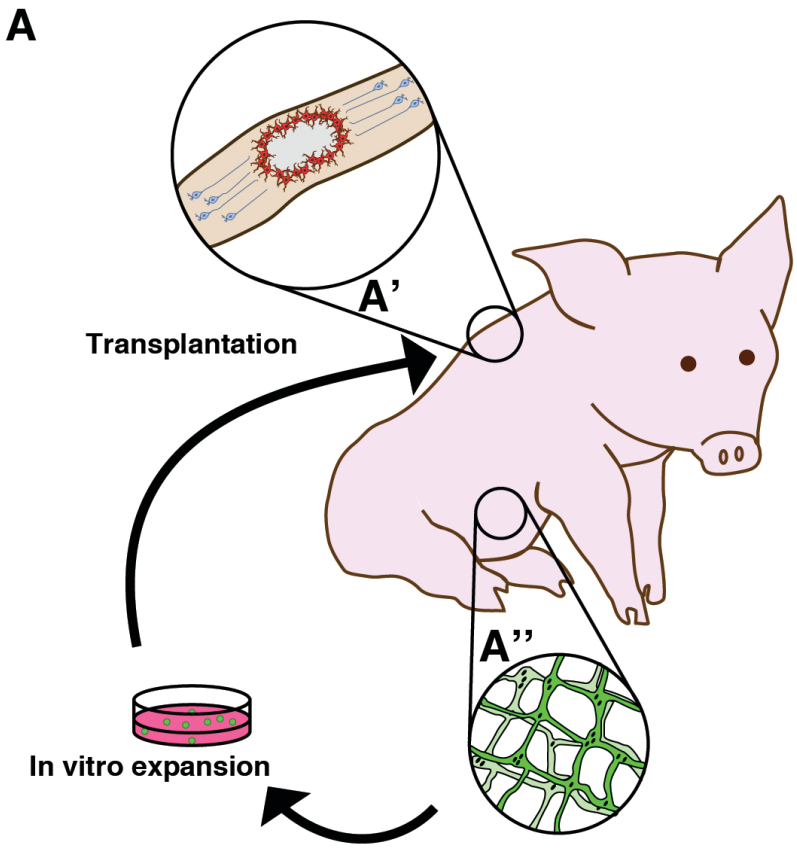

Figure 5.1: Large animal models for testing of autologous ENSC treatment.

To provide evidence that autologous transplantation of ENSCs is feasible, progression from rodent studies to a large animal model, such as the pig, will be required. Pigs are large enough to allow for the use of endoscopic biopsies, for harvesting ENSCs for SCI treatment. Following harvest from the ENS (A') ENSCs could be expanded in vitro and transplanted back into the injured spinal cord (A'') of the same animal. This approach would provide a platform to assess the effect of SCI on ENSC morphology, behaviour and proliferation /differentiation potential.

5.1.3.7 Benefits of ENSC cryopreservation

While it would be hoped that a single treatment with ENSCs/ ENSCs + ChABC would be sufficient to drive recovery following SCI, in order to achieve complete recovery it is probable that multiple treatments may be necessary at timed intervals to achieve success. In order to minimise invasiveness of these procedures, it would be advantageous to collect ENSCs from a single harvest of gut biopsies, expand these in culture and cryopreserve aliquots. These could then be thawed and expanded *in vitro* for any subsequent treatments. However, for such a strategy to be feasible it would need to be demonstrated that ENSCs can survive cryopreservation without any effect on cell viability. Several laboratories have noted decreases in stem cell viability after cell thawing, and so have focussed on increasing efficiency of cryopreserving stem cells (Berz et al., 2007; Hunt, 2011; Yuan et al., 2016b). Various methods have been optimised and could be tested to determine which offers the greatest stability to thawed cells. In particular, any effect of freezing that would affect their ability to enhance SCI repair would need to be assessed, including proliferative and differentiation capabilities. Demonstrating that cryopreservation has no negative effect on these properties would significantly increase the usability of ENSCs as a SCI therapy.

5.1.3.8 Additional safety assessments

As described above, results of the studies within this thesis raised no significant safety concerns of ENSC transplantation. However, future work will continue to assess this. In particular, analysis of ENSC proliferation post-transplantation will provide an important assessment of the potential of

ENSCs to form tumours, although previous work in our lab makes this unlikely (Cooper et al., 2016; McCann et al., 2017). Finally, an often-cited issue of iPSCs is the genetic instability arising from their generation (von Joest et al., 2016). Genetic sequencing should be conducted to confirm that this instability does not occur in ENSCs.

5.1.3.9 Use of ENSCs for additional nervous system disorders

As described above, this work and the work of others has demonstrated that ENSCs isolated from the ENS can be applied to CNS disorders. A useful future direction would be to explore other potential areas for ENSC application. Neurodegenerative diseases, for example, could be one such target. Parkinson's disease (PD) involves irreversible degeneration of dopaminergic neurons of the substantia nigra (Litvan et al., 2007). Stem cell therapy is arguably the most promising method to date for replacing these lost cells, and has shown promise in alleviating PD symptoms (Bjorklund, 2004). However, a previously successful trial utilising foetal stem cells is unlikely to be taken forward due to ethical concerns (Lindvall and Bjorklund, 2004), and other stem cell sources likely to be successful for PD will face many of the same issues as those proposed for SCI (evaluated in chapter 1). ENSCs may therefore serve as a viable alternative stem cell source for PD. Of note, any additional diseases would need to involve further, unique characterization of ENSCs. For example, PD is known to involve ENS complications, including an alpha-synuclein burden within enteric neurons (Awad, 2011; Pan-Montojo et al., 2010; Ulusoy et al., 2017; Wakabayashi et al., 1989). Therefore, ENSCs isolated from PD patients would need to be carefully compared to control (disease free) ENSCs to determine whether any deficits in the former would impair their ability to repopulate the substantia

nigra and other structures affected in PD. This may involve gene correction of isolated ENSCs prior to autologous transplantation, a process proposed in a review by Burns and Thapar, for ENSC-based treatments of enteric neuropathies such as Hirschsprung disease (Burns and Thapar, 2014) where mutations in the *RET* gene are prevalent. Thus, while an assessment of the suitability of ENSCs for additional nervous system disorders would likely yield promising results, it will be important to tailor ENSC preparation to the individual diseases.

5.1.3.10 The future of SCI therapies

As described in chapter 1, endogenous regeneration within the cord is unable to occur to any beneficial extent despite the fact that *in vitro* and *in vivo*, CNS regeneration has been demonstrated if the correct environment is supplied (Richardson et al., 1980; Taylor et al., 2005). This implies that modification of the inhibitory microenvironment is vital for SCI recovery. Previous publications have noted paracrine-driven stimulatory effects of transplanted stem cells on endogenous recovery (Baraniak and McDevitt, 2010), but it is likely that, even with these effects, several significant problems would remain. Arguably, one of the most prominent would be the dense concentration of CSPGs within the glial scar (Asher et al., 2001; Yuan and He, 2013). In this thesis therefore, ChABC was selected as a combinatorial therapy, based on the hypothesis that the combination of glial scar degradation, plus stimulation and bridge formation by transplanted ENSCs would allow endogenous axons to cross the injury site and re-establish previous connections. This expectation was supported by the effects of ENSC + ChABC treatment on reducing lesion parameters and increasing the number of retrograde axons crossing the injury site. Since the

commencement of this study, several other laboratories have published results from combinations of ChABC and other stem cells (Lee et al., 2015; Sarveazad et al., 2017; Suzuki et al., 2017). However, as with the current study, even these combined treatments do not completely nullify the inhibitory environment, and do not preserve all possible endogenous tissue, probably explaining the lack of complete recovery noticed in our and other studies. It is important to acknowledge that a complete SCI treatment will incorporate not only two, but most likely several individual methods. For example, the ENSC + ChABC treatment presented in chapter 4 begins 1 week after injury inducement. This transplantation time was selected based on a pilot study described in chapter 3 due to exclusion of ENSCs from the injury zone after this point. This transplantation time point could potentially be brought forward by pre-application of ChABC to prime the injury zone (see above). However, previous publications have shown in the early acute stages of SCI, the glial scar and even CSPGs may be beneficial (Rolls et al., 2009). Therefore, additional therapies are required to limit the damage sustained in these early stages that do not disrupt scar formation, and preferably that do not risk any further damage to the SC (minimizing SC injections etc.). One possibility is cooling of the lesion site. It has been shown that, shortly after SCI, many tracts survive the initial injury but become highly vulnerable and are lost in the acute phase. However, cooling of the spinal cord has shown that these tracts are still functional (James et al., 2011). The benefits of inducing mild hypothermia following SCI has been demonstrated in both human and animals (Dietrich et al., 2011). This therapy could be used in conjunction with the current ENSC + ChABC treatment, in the hours immediately following the injury but before transplantation. Moving into the initial days after the injury, anti-inflammatory drugs have been shown to have a neuroprotective effect at both acute and chronic stages of SCI (Arnold and

Hagg, 2011). Even ibuprofen has been shown to increase survival of spared tissue and lead to increased axonal elongation (Wang et al., 2009). This too could be easily utilised as a combinatorial therapy, after lesion cooling and before transplantation.

The works contained within this thesis show that the combined effects of transplanted ENSCs and ChABC-mediated degradation of CSPGs are highly beneficial, but neither treatment has any known effect towards other inhibitory debris of the injury zone, such as myelin fragments. However, as previously mentioned, a possible solution for these has already been uncovered (McGee and Strittmatter, 2003; O'Neill et al., 2004). Nogo antibodies have been shown to neutralise these myelin fragments, translating into significant improvements in motor recovery. This could be applied simultaneously with the ENSC/ChABC treatment, or could be administered in the week or so before ENSC transplantation. To reduce invasiveness of these multiple treatments, a systemic rather than spinal injection route could potentially be utilised, exploiting the reduced barrier function of the blood spinal cord barrier during SCI (Bartanusz et al., 2011; Whetstone et al., 2003).

Many other treatments have been shown to hold beneficial roles for SCI recovery, and many will likely prove to have significant benefit as part of a combinatorial therapy. A full treatment regimen is beyond the scope and purpose of this report, but the above points serve to demonstrate the potential for recovery if multiple treatments are to be combined. Numerous studies have demonstrated the benefits of combining two treatments (Bunge, 2016; Fuhrmann et al., 2017; Lu et al., 2004), but very few have examined the effects of combining multiple treatments (due largely to the rapidly expanding animal numbers, and costs, both financial and time, that accompany increasing numbers of variables). Despite this, such testing will be necessary before implementation of any combinatorial treatments, as in

rare cases certain combinations have proved counter-productive (Hoeber et al., 2017). In general however, results of such combinations are likely to be increasingly positive, and will undoubtedly form part of any future successful SCI treatment. Figure 5.2 shows a potential regimen by which several therapies could be beneficially combined to preserve the maximum endogenous tissue and optimise regeneration by transplanted ENSCs. It is worth noting however, that the odds of a single treatment being approved are incredibly small and subject to rigorous evaluation – a complex intervention such as the one proposed below is therefore incredibly unlikely to be accepted in the near future.

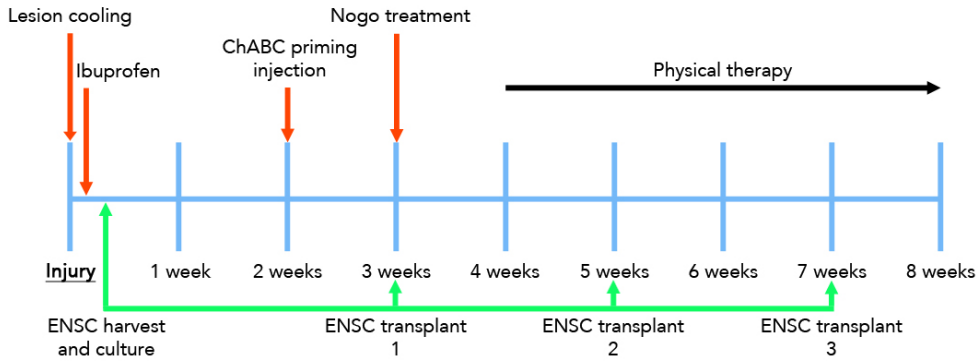

Figure 5.2: A proposed combined treatment regimen following SCI.

A successful future SCI treatment will likely combine multiple therapies to achieve full recovery. Following harvest of ENSCs once injured patients are medically stable, stem cells will be expanded in culture. It is likely that a single harvest will allow for several transplantations over time if required (green arrows). Simultaneously, non cell-based treatments (red arrows) will be applied to supplement and enhance the success of cell treatment. Finally, physical therapy following transplantation will likely prove vital for a full recovery.

5.1.4 Concluding remarks

The studies described within this thesis demonstrate the potential of stem cells isolated from the ENS to serve as an autologous cell-based source for not only enteric disorders, as previously described, but for nervous system disorders in general, and SCI specifically. The finding that ENSCs can survive and elicit therapeutic benefit following transplantation into the injured spinal cord provides proof of principle to allow ENSCs to be further tested for a range of neuropathies. In contrast to alternative stem cell sources proposed for SCI, ENSCs are easily and repeatedly accessible, with no significant ethical objections to their use and, as demonstrated in this thesis and other works, results of their transplantation has not warranted significant safety concerns. This work therefore supports the use of ENSCs as a novel stem cell source for patients with SCI. Future work will continue to elucidate the specific benefits of ENSC transplantation into the injured spinal cord.

Publications

Hockman D, Burns AJ, Schlosser G, Gates KP, **Jevans B**, Mongera A, Fisher S, Unlu G, Knapik EW, Kaufman CK, Mosimann C, Zon LI, Lancman JJ, Dong PDS, Lickert H, Tucker AS, Baker CVH, Evolution of the hypoxia-sensitive cells involved in amniote respiratory reflexes, *eLife*, 2017, Apr 7;6. pii: e21231. doi: 10.7554/eLife.21231.

McCann C, Cooper J, Natarajan D, **Jevans B**, Burns AJ, Thapar N, In vivo enteric nervous system stem cell transplantation restores functional responses in nNOS deficient colon, *Nature Communications*, 2017, Jul 3;8:15937. doi: 10.1038/ncomms15937.

Espinosa I, **Jevans B**, Boismoreau, Burns AJ, Brunet JF, Dual origin of the enteric nervous system in vagal Schwann cell precursors and sympathetic crest. Accepted, *Proceedings of the National Academy of Sciences USA*

Getreu N, **Jevans B**, Burns A, Fuller B, Hardiman P, Use of CAM assays to measure revascularization in ovarian tissue transplantation, In revision *Fertility and Sterility*

Jevans B, McCann C, Natarajan D, Cooper J, Thapar N, Burns AJ, Biology and therapeutic potential of enteric nervous system stem cells as a cell-based therapy for spinal cord injury. In preparation for *Journal of Anatomy*

Jevans B, James N, Burnside E, McCann C, Natarajan D, Cooper J, Thapar N, Bradbury E, Burns AJ, Going with your gut: the use of enteric nervous

system stem cells for spinal cord injury repair. In preparation for *Acta neuropathologica*

Bibliography

Abematsu, M., Tsujimura, K., Yamano, M., Saito, M., Kohno, K., Kohyama, J., Namihira, M., Komiya, S., Nakashima, K., 2010. Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. J Clin Invest 120, 3255-3266.

Adriaansen, J.J., Ruijs, L.E., van Koppenhagen, C.F., van Asbeck, F.W., Snoek, G.J., van Kuppevelt, D., Visser-Meily, J.M., Post, M.W., 2016. Secondary health conditions and quality of life in persons living with spinal cord injury for at least ten years. J Rehabil Med 48, 853-860.

Agbay, A., Edgar, J.M., Robinson, M., Styan, T., Wilson, K., Schroll, J., Ko, J., Khadem Mohtaram, N., Jun, M.B., Willerth, S.M., 2016. Biomaterial Strategies for Delivering Stem Cells as a Treatment for Spinal Cord Injury. Cells Tissues Organs 202, 42-51.

Anderson, A.J., Cummings, B.J., 2016. Achieving Informed Consent for Cellular Therapies: A Preclinical Translational Research Perspective on Regulations versus a Dose of Reality. J Law Med Ethics 44, 394-401.

Anderson, A.J., Haus, D.L., Hooshmand, M.J., Perez, H., Sontag, C.J., Cummings, B.J., 2011. Achieving stable human stem cell engraftment and survival in the CNS: is the future of regenerative medicine immunodeficient? Regen Med 6, 367-406.

Anderson, A.J., Piltti, K.M., Hooshmand, M.J., Nishi, R.A., Cummings, B.J., 2017. Preclinical Efficacy Failure of Human CNS-Derived Stem Cells for Use in the Pathway Study of Cervical Spinal Cord Injury. Stem Cell Reports 8, 249-263.

Anderson, K.D., 2004. Targeting recovery: priorities of the spinal cord-injured population. J Neurotrauma 21, 1371-1383.

Anderson, R.B., Newgreen, D.F., Young, H.M., 2006. Neural crest and the development of the enteric nervous system. Adv Exp Med Biol 589, 181-196.
Anderson, T.E., 1982. A controlled pneumatic technique for experimental spinal cord contusion. J Neurosci Methods 6, 327-333.
Ansari, A.M., Ahmed, A.K., Matsangos, A.E., Lay, F., Born, L.J., Marti, G., Harmon, J.W., Sun, Z., 2016. Cellular GFP Toxicity and Immunogenicity: Potential Confounders in in Vivo Cell Tracking Experiments. Stem Cell Rev 12, 553-559.
Antri, M., Barthe, J.Y., Mouffle, C., Orsal, D., 2005. Long-lasting recovery of locomotor function in chronic spinal rat following chronic combined pharmacological stimulation of serotonergic receptors with 8-OHDPAT and quipazine. Neurosci Lett 384, 162-167.
Arai, K., Harada, Y., Tomiyama, H., Michishita, M., Kanno, N., Yogo, T., Suzuki, Y., Hara, Y., 2016. Evaluation of the survival of bone marrow-derived mononuclear cells and the growth factors produced upon intramedullary transplantation in rat models of acute spinal cord injury. Res Vet Sci 107, 88-94.
Arnold, S.A., Hagg, T., 2011. Anti-inflammatory treatments during the chronic phase of spinal cord injury improve locomotor function in adult mice. J Neurotrauma 28, 1995-2002.
Asher, R.A., Morgenstern, D.A., Moon, L.D., Fawcett, J.W., 2001. Chondroitin sulphate proteoglycans: inhibitory components of the glial scar. Prog Brain Res 132, 611-619.
Assinck, P., Duncan, G.J., Hilton, B.J., Plemel, J.R., Tetzlaff, W., 2017. Cell transplantation therapy for spinal cord injury. Nat Neurosci 20, 637-647.
Auteri, M., Zizzo, M.G., Serio, R., 2015a. GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. Pharmacol Res 93, 11-21.

Auteri, M., Zizzo, M.G., Serio, R., 2015b. The GABAergic System and the Gastrointestinal Physiopathology. Curr Pharm Des 21, 4996-5016.
Awad, R.A., 2011. Neurogenic bowel dysfunction in patients with spinal cord injury, myelomeningocele, multiple sclerosis and Parkinson's disease. World J Gastroenterol 17, 5035-5048.
Bain, C.C., Bravo-Blas, A., Scott, C.L., Gomez Perdiguero, E., Geissmann, F., Henri, S., Malissen, B., Osborne, L.C., Artis, D., Mowat, A.M., 2014. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. Nat Immunol 15, 929-937.
Bain, C.C., Mowat, A.M., 2014. The monocyte-macrophage axis in the intestine. Cell Immunol 291, 41-48.
Baker, M., 2011. Stem-cell pioneer bows out. Nature 479, 459.
Ballios, B.G., Cooke, M.J., Donaldson, L., Coles, B.L., Morshead, C.M., van der Kooy, D., Shoichet, M.S., 2015. A Hyaluronan-Based Injectable Hydrogel Improves the Survival and Integration of Stem Cell Progeny following Transplantation. Stem Cell Reports 4, 1031-1045.
Bandtlow, C.E., Zimmermann, D.R., 2000. Proteoglycans in the developing brain: new conceptual insights for old proteins. Physiol Rev 80, 1267-1290.
Baraniak, P.R., McDevitt, T.C., 2010. Stem cell paracrine actions and tissue regeneration. Regen Med 5, 121-143.
Barbour, H.R., Plant, C.D., Harvey, A.R., Plant, G.W., 2013. Tissue sparing, behavioral recovery, supraspinal axonal sparing/regeneration following sub-acute glial transplantation in a model of spinal cord contusion. BMC Neurosci 14, 106.
Barnabe-Heider, F., Goritz, C., Sabelstrom, H., Takebayashi, H., Pfrieger, F.W., Meletis, K., Frisen, J., 2010. Origin of new glial cells in intact and injured adult spinal cord. Cell Stem Cell 7, 470-482.

Barnett, S.C., Riddell, J.S., 2004. Olfactory ensheathing cells (OECs) and the treatment of CNS injury: advantages and possible caveats. J Anat 204, 57-67.
Barraud, P., Seferiadis, A.A., Tyson, L.D., Zwart, M.F., Szabo-Rogers, H.L., Ruhrberg, C., Liu, K.J., Baker, C.V., 2010. Neural crest origin of olfactory ensheathing glia. Proc Natl Acad Sci U S A 107, 21040-21045.
Bartanusz, V., Jezova, D., Alajajian, B., Digicaylioglu, M., 2011. The blood-spinal cord barrier: morphology and clinical implications. Ann Neurol 70, 194-206.
Bartus, K., James, N.D., Bosch, K.D., Bradbury, E.J., 2012. Chondroitin sulphate proteoglycans: key modulators of spinal cord and brain plasticity. Exp Neurol 235, 5-17.
Bartus, K., James, N.D., Didangelos, A., Bosch, K.D., Verhaagen, J., Yanez-Munoz, R.J., Rogers, J.H., Schneider, B.L., Muir, E.M., Bradbury, E.J., 2014. Large-scale chondroitin sulfate proteoglycan digestion with chondroitinase gene therapy leads to reduced pathology and modulates macrophage phenotype following spinal cord contusion injury. J Neurosci 34, 4822-4836.
Bastidas, J., Athauda, G., De La Cruz, G., Chan, W.M., Golshani, R., Berrocal, Y., Henao, M., Lalwani, A., Mannoji, C., Assi, M., Otero, P.A., Khan, A., Marcillo, A.E., Norenberg, M., A, D.L., Wood, P.M., Guest, J.D., Dietrich, W.D., Bartlett Bunge, M., Pearse, D.D., 2017. Human schwann cells exhibit long-term cell survival, are not tumorigenic and promote repair when transplanted into the contused spinal cord. Glia.
Bayliss, W.M., Starling, E.H., 1899. The Movements and Innervation of the Small Intestine. J Physiol 24, 99–143.
Beattie, M.S., Bresnahan, J.C., Komon, J., Tovar, C.A., Van Meter, M., Anderson, D.K., Faden, A.I., Hsu, C.Y., Noble, L.J., Salzman, S., Young, W., 1997. Endogenous repair after spinal cord contusion injuries in the rat. Exp Neurol 148, 453-463.

Beck, K.D., Nguyen, H.X., Galvan, M.D., Salazar, D.L., Woodruff, T.M., Anderson, A.J., 2010. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. Brain 133, 433-447.

Belkind-Gerson, J., Hotta, R., Whalen, M., Nayyar, N., Nagy, N., Cheng, L., Zuckerman, A., Goldstein, A.M., Dietrich, J., 2016. Engraftment of enteric neural progenitor cells into the injured adult brain. BMC Neurosci 17, 5.

Bellairs, R., Osmond, M., 1998. The atlas of chick development. San Diego: Academic Press.

Belmadani, A., Tran, P.B., Ren, D., Assimacopoulos, S., Grove, E.A., Miller, R.J., 2005. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25, 3995-4003.

Ben Haim, L., Carrillo-de Sauvage, M.A., Ceyzeriat, K., Escartin, C., 2015. Elusive roles for reactive astrocytes in neurodegenerative diseases. Front Cell Neurosci 9, 278.

Berz, D., McCormack, E.M., Winer, E.S., Colvin, G.A., Quesenberry, P.J., 2007. Cryopreservation of hematopoietic stem cells. Am J Hematol 82, 463-472.

Binder, E., Natarajan, D., Cooper, J., Kronfli, R., Cananzi, M., Delalande, J.M., McCann, C., Burns, A.J., Thapar, N., 2015. Enteric neurospheres are not specific to neural crest cultures: implications for neural stem cell therapies. PLoS One 10, e0119467.

Bjorklund, L.M., 2004. Stem cell therapy for Parkinson's disease. Dialogues Clin Neurosci 6, 303-311.

Boido, M., Garbossa, D., Fontanella, M., Ducati, A., Vercelli, A., 2014. Mesenchymal stem cell transplantation reduces glial cyst and improves functional outcome after spinal cord compression. World Neurosurg 81, 183-190.

Bondurand, N., Natarajan, D., Barlow, A., Thapar, N., Pachnis, V., 2006. Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling. Development 133, 2075-2086.

Bondurand, N., Natarajan, D., Thapar, N., Atkins, C., Pachnis, V., 2003. Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures. Development 130, 6387-6400.

Borody, T.J., Quigley, E.M., Phillips, S.F., Wienbeck, M., Tucker, R.L., Haddad, A., Zinsmeister, A.R., 1985. Effects of morphine and atropine on motility and transit in the human ileum. Gastroenterology 89, 562-570.

Bradbury, E.J., Moon, L.D., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W., McMahon, S.B., 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416, 636-640.

Bradl, M., Lassmann, H., 2010. Oligodendrocytes: biology and pathology. Acta Neuropathol 119, 37-53.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C., Riethmacher, D., 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev 12, 1825-1836.

Bronner-Fraser, M., 1994. Neural crest cell formation and migration in the developing embryo. FASEB J 8, 699-706.

Bronner-Fraser, M., Fraser, S.E., 1997. Differentiation of the vertebrate neural tube. Curr Opin Cell Biol 9, 885-891.

Brösamle, C., Huber, A., 2006. Cracking the black box – and putting it back together again: Animal models of spinal cord injury,. Drug Discovery Today: Disease Models 3, 341-347.

Brown, D.S., Eames, B.F., 2016. Emerging tools to study proteoglycan function during skeletal development. Methods Cell Biol 134, 485-530.

Browning, K.N., Verheijden, S., Boeckxstaens, G.E., 2017. The Vagus Nerve in Appetite Regulation, Mood, and Intestinal Inflammation. Gastroenterology 152, 730-744.
Bulbring, E., Burn, J.H., 1941. Observations bearing on synaptic transmission by acetylcholine in the spinal cord. J Physiol 100, 337-368.
Bulbring, E., Crema, A., 1958. Observations concerning the action of 5-hydroxytryptamine on the peristaltic reflex. Br J Pharmacol Chemother 13, 444-457.
Bulbring, E., Lin, R.C., 1958. The effect of intraluminal application of 5-hydroxytryptamine and 5-hydroxytryptophan on peristalsis; the local production of 5-HT and its release in relation to intraluminal pressure and propulsive activity. J Physiol 140, 381-407.
Bult, H., Boeckxstaens, G.E., Pelckmans, P.A., Jordaens, F.H., Van Maercke, Y.M., Herman, A.G., 1990. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345, 346-347.
Bunge, M.B., 2016. Efficacy of Schwann cell transplantation for spinal cord repair is improved with combinatorial strategies. J Physiol 594, 3533-3538.
Burns, A., Thapar, N., 2014. Neural stem cell therapies for enteric nervous system disorders. Nat Rev Gastroenterol Hepatol 11, 317-328.
Burns, A.J., Delalande, J.M., Le Douarin, N.M., 2002. In ovo transplantation of enteric nervous system precursors from vagal to sacral neural crest results in extensive hindgut colonisation. Development 129, 2785-2796.
Burns, A.J., Douarin, N.M., 1998. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. Development 125, 4335-4347.
Burns, A.J., Goldstein, A.M., Newgreen, D.F., Stamp, L., Schafer, K.H., Metzger, M., Hotta, R., Young, H.M., Andrews, P.W., Thapar, N., Belkind-Gerson, J., Bondurand, N., Bornstein, J.C., Chan, W.Y., Cheah, K., Gershon,

M.D., Heuckeroth, R.O., Hofstra, R.M., Just, L., Kapur, R.P., King, S.K., McCann, C.J., Nagy, N., Ngan, E., Obermayr, F., Pachnis, V., Pasricha, P.J., Sham, M.H., Tam, P., Vanden Berghe, P., 2016. White paper on guidelines concerning enteric nervous system stem cell therapy for enteric neuropathies. Dev Biol 417, 229-251.

Burns, A.J., Le Douarin, N.M., 1998. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. Development 125, 4335-4347.

Burns, A.J., Le Douarin, N.M., 2001. Enteric nervous system development: Analysis of the selective developmental potentialities of vagal and sacral neural crest cells using quail-chick chimeras. The Anatomical Record 262, 16-28.

Burnside, E.R., Bradbury, E.J., 2014. Manipulating the extracellular matrix and its role in brain and spinal cord plasticity and repair. Neuropathol Appl Neurobiol 40, 26-59.

Burt, D.W., 2007. Emergence of the chicken as a model organism: implications for agriculture and biology. Poult Sci 86, 1460-1471.

Caggiano, A.O., Zimber, M.P., Ganguly, A., Blight, A.R., Gruskin, E.A., 2005. Chondroitinase ABCI improves locomotion and bladder function following contusion injury of the rat spinal cord. J Neurotrauma 22, 226-239.

Cao, Y., Massaro, J.F., Krause, J.S., Chen, Y., Devivo, M.J., 2014. Suicide mortality after spinal cord injury in the United States: injury cohorts analysis. Arch Phys Med Rehabil 95, 230-235.

Carlson, S.L., Parrish, M.E., Springer, J.E., Doty, K., Dossett, L., 1998. Acute inflammatory response in spinal cord following impact injury. Exp Neurol 151, 77-88.

Caspary, T., Anderson, K.V., 2003. Patterning cell types in the dorsal spinal cord: what the mouse mutants say. Nat Rev Neurosci 4, 289-297.

Cheng, L.S., Hotta, R., Graham, H.K., Belkind-Gerson, J., Nagy, N., Goldstein, A.M., 2017. Postnatal human enteric neuronal progenitors can migrate, differentiate, and proliferate in embryonic and postnatal aganglionic gut environments. Pediatr Res 81, 838-846.

Cheriyan, T., Ryan, D.J., Weinreb, J.H., Cheriyan, J., Paul, J.C., Lafage, V., Kirsch, T., Errico, T.J., 2014. Spinal cord injury models: a review. Spinal Cord 52, 588-595.

Chey, W.Y., Gutierrez, J.G., 1978. The endocrine control of gastrointestinal function. Adv Intern Med 23, 61-84.

Church, J.S., Milich, L.M., Lerch, J.K., Popovich, P.G., McTigue, D.M., 2017. E6020, a synthetic TLR4 agonist, accelerates myelin debris clearance, Schwann cell infiltration, and remyelination in the rat spinal cord. Glia 65, 883-899.

Cirillo, G., De Luca, D., Papa, M., 2012. Calcium imaging of living astrocytes in the mouse spinal cord following sensory stimulation. Neural Plast 2012, 425818.

Clearwaters, K., 1954. Regeneration of the spinal cord of the chick. J Comp Neurol 101, 317–329.

Colas, J.F., Schoenwolf, G.C., 2001. Towards a cellular and molecular understanding of neurulation. Dev Dyn 221, 117-145.

Cooper, J.E., McCann, C.J., Natarajan, D., Choudhury, S., Boesmans, W., Delalande, J.M., Vanden Berghe, P., Burns, A.J., Thapar, N., 2016. In Vivo Transplantation of Enteric Neural Crest Cells into Mouse Gut; Engraftment, Functional Integration and Long-Term Safety. PLoS One 11, e0147989.

Cooper, J.E., Natarajan, D., McCann, C.J., Choudhury, S., Godwin, H., Burns, A.J., Thapar, N., 2017. In vivo transplantation of fetal human gut-derived enteric neural crest cells. Neurogastroenterol Motil 29.

Corns, L.F., Atkinson, L., Daniel, J., Edwards, I.J., New, L., Deuchars, J., Deuchars, S.A., 2015. Cholinergic Enhancement of Cell Proliferation in the Postnatal Neurogenic Niche of the Mammalian Spinal Cord. Stem Cells 33, 2864-2876.

Costa, M., Brookes, S.J., Hennig, G.W., 2000a. Anatomy and physiology of the enteric nervous system. Gut 47 Suppl 4, iv15-19; discussion iv26.

Costa, M., Glise, H., Sjodahl, R., 2000b. The enteric nervous system in health and disease. Gut 47, iv1.

Cote, M.P., Amin, A.A., Tom, V.J., Houle, J.D., 2011. Peripheral nerve grafts support regeneration after spinal cord injury. Neurotherapeutics 8, 294-303.

Craig, A., Guest, R., Tran, Y., Middleton, J., 2017. Depressive mood in adults with spinal cord injury as they transition from an inpatient to a community setting: secondary analyses from a clinical trial. Spinal Cord.

Crespo, D., Asher, R.A., Lin, R., Rhodes, K.E., Fawcett, J.W., 2007. How does chondroitinase promote functional recovery in the damaged CNS? Exp Neurol 206, 159-171.

Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., Anderson, A.J., 2005. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A 102, 14069-14074.

Curtis, D.R., Phillis, J.W., Watkins, J.C., 1960. The chemical excitation of spinal neurones by certain acidic amino acids. J Physiol 150, 656-682.

Cusimano, M., Biziato, D., Brambilla, E., Donega, M., Alfaro-Cervello, C., Snider, S., Salani, G., Pucci, F., Comi, G., Garcia-Verdugo, J.M., De Palma, M., Martino, G., Pluchino, S., 2012. Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. Brain 135, 447-460.

Dai, G., Liu, X., Zhang, Z., Yang, Z., Dai, Y., Xu, R., 2013. Transplantation of autologous bone marrow mesenchymal stem cells in the treatment of complete and chronic cervical spinal cord injury. Brain Res 1533, 73-79.
Dale, H., 1937. Transmission of Nervous Effects by Acetylcholine: Harvey Lecture, May 20, 1937. Bull N Y Acad Med 13, 379-396.
Dasari, V.R., Veeravalli, K.K., Dinh, D.H., 2014. Mesenchymal stem cells in the treatment of spinal cord injuries: A review. World J Stem Cells 6, 120-133.
David, S., Kroner, A., 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nat Rev Neurosci 12, 388-399.
Decimo, I., Bifari, F., Krampera, M., Fumagalli, G., 2012. Neural stem cell niches in health and diseases. Curr Pharm Des 18, 1755-1783.
Delalande, J.M., Thapar, N., Burns, A.J., 2015. Dual labeling of neural crest cells and blood vessels within chicken embryos using Chick(GFP) neural tube grafting and carbocyanine dye DiI injection. J Vis Exp, e52514.
Demaison, C., Parsley, K., Brouns, G., Scherr, M., Battmer, K., Kinnon, C., Grez, M., Thrasher, A.J., 2002. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther 13, 803-813.
den Braber-Ymker, M., Lammens, M., van Putten, M.J., Nagtegaal, I.D., 2017. The enteric nervous system and the musculature of the colon are altered in patients with spina bifida and spinal cord injury. Virchows Arch 470, 175-184.
Devaux, S., Cizkova, D., Quanico, J., Franck, J., Nataf, S., Pays, L., Hauberg-Lotte, L., Maass, P., Kobarg, J.H., Kobeissy, F., Meriaux, C., Wisztorski, M., Slovinska, L., Blasko, J., Cigankova, V., Fournier, I., Salzet, M., 2016. Proteomic Analysis of the Spatio-temporal Based Molecular Kinetics of Acute

Spinal Cord Injury Identifies a Time- and Segment-specific Window for Effective Tissue Repair. Mol Cell Proteomics 15, 2641-2670.
Dietrich, W.D., Levi, A.D., Wang, M., Green, B.A., 2011. Hypothermic treatment for acute spinal cord injury. Neurotherapeutics 8, 229-239.
Dikshit, B.B., 1934. Action of acetylcholine on the brain and its occurrence therein. J Physiol 80, 409-421.
Donaldson, A.E., Cai, J., Yang, M., Iacovitti, L., 2009. Human amniotic fluid stem cells do not differentiate into dopamine neurons in vitro or after transplantation in vivo. Stem Cells Dev 18, 1003-1012.
Donnelly, E.M., Lamanna, J., Boulis, N.M., 2012. Stem cell therapy for the spinal cord. Stem Cell Res Ther 3, 24.
Dulin, J.N., Lu, P., 2014. Bridging the injured spinal cord with neural stem cells. Neural Regen Res 9, 229-231.
Edmondson, R., Broglie, J.J., Adcock, A.F., Yang, L., 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol 12, 207-218.
Fan, X., Wang, J.Z., Lin, X.M., Zhang, L., 2017. Stem cell transplantation for spinal cord injury: a meta-analysis of treatment effectiveness and safety. Neural Regen Res 12, 815-825.
Faulkner, J., Keirstead, H.S., 2005. Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol 15, 131-142.
Fawcett, J.W., 2008. Bridging spinal cord injuries. J Biol 7, 25.
Fawcett, J.W., 2015. The extracellular matrix in plasticity and regeneration after CNS injury and neurodegenerative disease. Prog Brain Res 218, 213-226.
Fawcett, J.W., Asher, R.A., 1999. The glial scar and central nervous system repair. Brain Res Bull 49, 377-391.

Fehlings, M.G., Vawda, R., 2011. Cellular treatments for spinal cord injury: the time is right for clinical trials. Neurotherapeutics 8, 704-720.
Ferretti, P., Whalley, K., 2008. Successful neural regeneration in amniotes: the developing chick spinal cord. Cell Mol Life Sci 65, 45-53.
Ferretti, P., Zhang, F., O'Neill, P., 2003. Changes in spinal cord regenerative ability through phylogenesis and development: lessons to be learnt. Dev Dyn 226, 245-256.
Fleming, J.C., Norenberg, M.D., Ramsay, D.A., Dekaban, G.A., Marcillo, A.E., Saenz, A.D., Pasquale-Styles, M., Dietrich, W.D., Weaver, L.C., 2006. The cellular inflammatory response in human spinal cords after injury. Brain 129, 3249-3269.
Fouad, K., Hurd, C., Magnuson, D.S., 2013. Functional testing in animal models of spinal cord injury: not as straight forward as one would think. Front Integr Neurosci 7, 85.
Fouad, K., Schnell, L., Bunge, M.B., Schwab, M.E., Liebscher, T., Pearse, D.D., 2005. Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. J Neurosci 25, 1169-1178.
Freem, L.J., Delalande, J.M., Campbell, A.M., Thapar, N., Burns, A.J., 2012. Lack of organ specific commitment of vagal neural crest cell derivatives as shown by back-transplantation of GFP chicken tissues. Int J Dev Biol 56, 245-254.
Frieling, T., Wood, J.D., Cooke, H.J., 1992. Submucosal reflexes: distension-evoked ion transport in the guinea pig distal colon. Am J Physiol 263, G91-96.
Frischknecht, R., Seidenbecher, C.I., 2008. The crosstalk of hyaluronan-based extracellular matrix and synapses. Neuron Glia Biol 4, 249-257.

Fuhrmann, T., Anandakumaran, P.N., Shoichet, M.S., 2017. Combinatorial Therapies After Spinal Cord Injury: How Can Biomaterials Help? Adv Healthc Mater 6.

Fuhrmann, T., Tam, R.Y., Ballarin, B., Coles, B., Elliott Donaghue, I., van der Kooy, D., Nagy, A., Tator, C.H., Morshead, C.M., Shoichet, M.S., 2016. Injectable hydrogel promotes early survival of induced pluripotent stem cell-derived oligodendrocytes and attenuates longterm teratoma formation in a spinal cord injury model. Biomaterials 83, 23-36.

Furness, J.B., 2000. Types of neurons in the enteric nervous system. J Auton Nerv Syst 81, 87-96.

Furness, J.B., 2006. The enteric nervous system. Blackwell Publishing; Oxford.

Furness, J.B., 2012. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 9, 286-294.

Gall, A., Turner-Stokes, L., 2008. Chronic spinal cord injury: management of patients in acute hospital settings. Clin Med 8, 70–74.

Gammill, L.S., Bronner-Fraser, M., 2003. Neural crest specification: migrating into genomics. Nat Rev Neurosci 4, 795-805.

Garthwaite, J., Charles, S.L., Chess-Williams, R., 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336, 385-388.

Gattlen, C., Clarke, C.B., Piller, N., Kirschmann, G., Pertin, M., Decosterd, I., Gosselin, R.D., Suter, M.R., 2016. Spinal Cord T-Cell Infiltration in the Rat Spared Nerve Injury Model: A Time Course Study. Int J Mol Sci 17, 352.

Geissmann, F., Gordon, S., Hume, D.A., Mowat, A.M., Randolph, G.J., 2010a. Unravelling mononuclear phagocyte heterogeneity. Nat Rev Immunol 10, 453-460.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., Ley, K., 2010b. Development of monocytes, macrophages, and dendritic cells. Science 327, 656-661.
Gensel, J.C., Tovar, C.A., Hamers, F.P., Deibert, R.J., Beattie, M.S., Bresnahan, J.C., 2006. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. J Neurotrauma 23, 36-54.
Gershon, M.D., 2013. 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. Curr Opin Endocrinol Diabetes Obes 20, 14-21.
Gershon, M.D., Chalazonitis, A., Rothman, T.P., 1993. From neural crest to bowel: development of the enteric nervous system. J Neurobiol 24, 199-214.
Gershon, M.D., Rothman, T.P., 1991. Enteric glia. Glia 4, 195-204.
Gershon, M.D., Tack, J., 2007. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 132, 397-414.
Gilbert, S.F., 2000. Paradigm shifts in neural induction. Rev Hist Sci Paris 53, 555-579.
Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., Samokhvalov, I.M., Merad, M., 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330, 841-845.
Goldstein, A.M., Hofstra, R.M., Burns, A.J., 2013. Building a brain in the gut: development of the enteric nervous system. Clin Genet 83, 307-316.
Goldstein, E.Z., Church, J.S., Hesp, Z.C., Popovich, P.G., McTigue, D.M., 2016. A silver lining of neuroinflammation: Beneficial effects on myelination. Exp Neurol 283, 550-559.

Gonzalez-Perez, O., Lopez-Virgen, V., Quinones-Hinojosa, A., 2015. Astrocytes: Everything but the glue. Neuroimmunol Neuroinflamm 2, 115-117.
Gorustovich, A.A., Roether, J.A., Boccaccini, A.R., 2010. Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. Tissue Eng Part B Rev 16, 199-207.
Gouti, M., Metzis, V., Briscoe, J., 2015. The route to spinal cord cell types: a tale of signals and switches. Trends Genet 31, 282-289.
Grumet, M., Flaccus, A., Margolis, R.U., 1993. Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules. J Cell Biol 120, 815-824.
Guest, J.D., Hiester, E.D., Bunge, R.P., 2005. Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury. Exp Neurol 192, 384-393.
Guizar-Sahagun, G., Garcia-Lopez, P., Espitia, A.L., Mendez, S., Castaneda-Hernandez, G., Madrazo, I., Franco-Bourland, R.E., 1996. Histochemical evidence of the increased expression of nicotinamide adenine dinucleotide phosphate-dependent diaphorase in neurons of the myenteric plexus after acute spinal cord injury in adult rats. Neurosci Lett 206, 185-188.
Gulbransen, B.D., Sharkey, K.A., 2012. Novel functional roles for enteric glia in the gastrointestinal tract. Nat Rev Gastroenterol Hepatol 9, 625-632.
Guo, D., Hu, J., 2014. Spinal presynaptic inhibition in pain control. Neuroscience 283, 95-106.
Gwak, Y.S., Kang, J., Unabia, G.C., Hulsebosch, C.E., 2012. Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. Exp Neurol 234, 362-372.

Gwak, Y.S., Tan, H.Y., Nam, T.S., Paik, K.S., Hulsebosch, C.E., Leem, J.W., 2006. Activation of spinal GABA receptors attenuates chronic central neuropathic pain after spinal cord injury. J Neurotrauma 23, 1111-1124.

Haas, B., Playford, E.D., Ahmad, A.Q., Yildiran, T., Gibbon, A.J., Freeman, J.A., 2016. Rehabilitation goals of people with spinal cord injuries can be classified against the International Classification of Functioning, Disability and Health Core Set for spinal cord injuries. Spinal Cord 54, 324-328.

Hachem, L.D., Ahuja, C.S., Fehlings, M.G., 2017. Assessment and management of acute spinal cord injury: From point of injury to rehabilitation. J Spinal Cord Med, 1-11.

Hains, B.C., Saab, C.Y., Lo, A.C., Waxman, S.G., 2004. Sodium channel blockade with phenytoin protects spinal cord axons, enhances axonal conduction, and improves functional motor recovery after contusion SCI. Exp Neurol 188, 365-377.

Hamada, Y., Ikata, T., Katoh, S., Tsuchiya, K., Niwa, M., Tsutsumishita, Y., Fukuzawa, K., 1996. Roles of nitric oxide in compression injury of rat spinal cord. Free Radic Biol Med 20, 1-9.

Hao, M.M., Anderson, R.B., Kobayashi, K., Whitington, P.M., Young, H.M., 2009. The migratory behavior of immature enteric neurons. Dev Neurobiol 69, 22-35.

Hara, M., Kobayakawa, K., Ohkawa, Y., Kumamaru, H., Yokota, K., Saito, T., Kijima, K., Yoshizaki, S., Harimaya, K., Nakashima, Y., Okada, S., 2017. Interaction of reactive astrocytes with type I collagen induces astrocytic scar formation through the integrin-N-cadherin pathway after spinal cord injury. Nat Med 23, 818-828.

Hartline, D.K., Colman, D.R., 2007. Rapid conduction and the evolution of giant axons and myelinated fibers. Curr Biol 17, R29-35.

Harvey, W., 1628. Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus.

Hasan, S., Keirstead, H., Muir, G., Steeves, J., 1993. Axonal Regeneration Contributes to Repair of Injured Brainstem-Spinal Neurons in Embryonic Chick. Journal of Neuroscience 13, 492-507.

Hasan, S.J., Nelson, B.H., Valenzuela, J.I., Keirstead, H.S., Shull, S.E., Ethell, D.W., Steeves, J.D., 1991. Functional repair of transected spinal cord in embryonic chick. Restor Neurol Neurosci 2, 137-154.

Hayashibe, M., Homma, T., Fujimoto, K., Oi, T., Yagi, N., Kashihara, M., Nishikawa, N., Ishizumi, Y., Abe, S., Hashimoto, H., Kanekiyo, K., Imagita, H., Ide, C., Morioka, S., 2016. Locomotor improvement of spinal cord-injured rats through treadmill training by forced plantar placement of hind paws. Spinal Cord 54, 521-529.

Hayden, E.C., 2014. Funding windfall rescues abandoned stem-cell trial. Nature 510, 18.

Heanue, T.A., Pachnis, V., 2007. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nat Rev Neurosci 8, 466-479.

Heinicke, E.A., Kiernan, J.A., 1990. An immunohistochemical study of the myenteric plexus of the colon in the rat and mouse. J Anat 170, 51-62.

Henderson, D.J., Copp, A.J., 1997. Role of the extracellular matrix in neural crest cell migration. J Anat 191 (Pt 4), 507-515.

Henningsen, J., Rigbolt, K.T., Blagoev, B., Pedersen, B.K., Kratchmarova, I., 2010. Dynamics of the skeletal muscle secretome during myoblast differentiation. Mol Cell Proteomics 9, 2482-2496.

Herlenius, E., Lagercrantz, H., 2004. Development of neurotransmitter systems during critical periods. Exp Neurol 190 Suppl 1, S8-21.

Hirano, S., Norita, M., Hoshino, K., Meguro, R., 1996. Morphological analysis of the early development of the chick neural tube separated from the floor plate and notochord. Exp Neurol 139, 317-321.

Hirota, T., Ohta, H., Powell, B.E., Mahadevaiah, S.K., Ojarikre, O.A., Saitou, M., Turner, J.M.A., 2017. Fertile offspring from sterile sex chromosome trisomic mice. Science 357, 932-935.

Hoeber, J., Konig, N., Trolle, C., Lekholm, E., Zhou, C., Pankratova, S., Akesson, E., Fredriksson, R., Aldskogius, H., Kozlova, E.N., 2017. A Combinatorial Approach to Induce Sensory Axon Regeneration into the Dorsal Root Avulsed Spinal Cord. Stem Cells Dev 26, 1065-1077.

Holmes, G.M., 2012. Upper gastrointestinal dysmotility after spinal cord injury: is diminished vagal sensory processing one culprit? Front Physiol 3, 277.

Hong, J.Y., Lee, S.H., Lee, S.C., Kim, J.W., Kim, K.P., Kim, S.M., Tapia, N., Lim, K.T., Kim, J., Ahn, H.S., Ko, K., Shin, C.Y., Lee, H.T., Scholer, H.R., Hyun, J.K., Han, D.W., 2014. Therapeutic potential of induced neural stem cells for spinal cord injury. J Biol Chem 289, 32512-32525.

Horii-Hayashi, N., Sasagawa, T., Matsunaga, W., Nishi, M., 2015. Development and Structural Variety of the Chondroitin Sulfate Proteoglycans-Contained Extracellular Matrix in the Mouse Brain. Neural Plast 2015, 256389.

Hou, S., Carson, D.M., Wu, D., Klaw, M.C., Houle, J.D., Tom, V.J., 2016. Dopamine is produced in the rat spinal cord and regulates micturition reflex after spinal cord injury. Exp Neurol 285, 136-146.

Hou, S., Rabchevsky, A.G., 2014. Autonomic consequences of spinal cord injury. Compr Physiol 4, 1419-1453.

Hu, B.Y., Zhang, S.C., 2009. Differentiation of spinal motor neurons from pluripotent human stem cells. Nat Protoc 4, 1295-1304.

Huebner, E.A., Strittmatter, S.M., 2009. Axon regeneration in the peripheral and central nervous systems. Results Probl Cell Differ 48, 339-351.
Hunt, C.J., 2011. Cryopreservation of Human Stem Cells for Clinical Application: A Review. Transfus Med Hemother 38, 107-123.
Husch, A., Van Patten, G.N., Hong, D.N., Scaperotti, M.M., Cramer, N., Harris-Warrick, R.M., 2012. Spinal cord injury induces serotonin supersensitivity without increasing intrinsic excitability of mouse V2a interneurons. J Neurosci 32, 13145-13154.
Huttner, W.B., Kosodo, Y., 2005. Symmetric versus asymmetric cell division during neurogenesis in the developing vertebrate central nervous system. Curr Opin Cell Biol 17, 648-657.
Hyatt, A.J., Wang, D., van Oterendorp, C., Fawcett, J.W., Martin, K.R., 2014. Mesenchymal stromal cells integrate and form longitudinally-aligned layers when delivered to injured spinal cord via a novel fibrin scaffold. Neurosci Lett 569, 12-17.
Iaci, J.F., Vecchione, A.M., Zimber, M.P., Caggiano, A.O., 2007. Chondroitin sulfate proteoglycans in spinal cord contusion injury and the effects of chondroitinase treatment. J Neurotrauma 24, 1743-1759.
Ide, C., Kanekiyo, K., 2016. Points regarding cell transplantation for the treatment of spinal cord injury. Neural Regen Res 11, 1046-1049.
Inman, D., Guth, L., Steward, O., 2002. Genetic influences on secondary degeneration and wound healing following spinal cord injury in various strains of mice. J Comp Neurol 451, 225-235.
Isomoto, S., Hattori, K., Ohgushi, H., Nakajima, H., Tanaka, Y., Takakura, Y., 2007. Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells. J Orthop Sci 12, 83-88.
Jacobs, B.L., Fornal, C.A., 1997. Serotonin and motor activity. Curr Opin Neurobiol 7, 820-825.

Jacobs, B.L., Martin-Cora, F.J., Fornal, C.A., 2002. Activity of medullary serotonergic neurons in freely moving animals. Brain Res Brain Res Rev 40, 45-52.

Jaeger, C., Toombs, J., Borgens, R., 1993. Grafting in Acute Spinal Cord injury: Morphological and Immunological Aspects of Transplanted Adult Rat Enteric Ganglia Neuroscience 52, 333-346.

James, N.D., Bartus, K., Grist, J., Bennett, D.L., McMahon, S.B., Bradbury, E.J., 2011. Conduction failure following spinal cord injury: functional and anatomical changes from acute to chronic stages. J Neurosci 31, 18543-18555.

James, N.D., Shea, J., Muir, E.M., Verhaagen, J., Schneider, B.L., Bradbury, E.J., 2015. Chondroitinase gene therapy improves upper limb function following cervical contusion injury. Exp Neurol 271, 131-135.

Jessen, K.R., Hills, J.M., Dennison, M.E., Mirsky, R., 1983. Gamma-aminobutyrate as an autonomic neurotransmitter: release and uptake of [3H]gamma-aminobutyrate in guinea pig large intestine and cultured enteric neurons using physiological methods and electron microscopic autoradiography. Neuroscience 10, 1427-1442.

Jessen, K.R., Mirsky, R., Dennison, M.E., Burnstock, G., 1979. GABA may be a neurotransmitter in the vertebrate peripheral nervous system. Nature 281, 71-74.

Jiang, X., Rowitch, D.H., Soriano, P., McMahon, A.P., Sucov, H.M., 2000. Fate of the mammalian cardiac neural crest. Development. 127, 1607-1616.

Jones, C.F., Lee, J.H., Kwon, B.K., Cripton, P.A., 2012. Development of a large-animal model to measure dynamic cerebrospinal fluid pressure during spinal cord injury: Laboratory investigation. J Neurosurg Spine 16, 624-635.

Kadoya, K., Lu, P., Nguyen, K., Lee-Kubli, C., Kumamaru, H., Yao, L., Knackert, J., Poplawski, G., Dulin, J.N., Strobl, H., Takashima, Y., Biane, J.,

Conner, J., Zhang, S.C., Tuszynski, M.H., 2016. Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. Nat Med 22, 479-487.
Kanagal, S.G., Muir, G.D., 2007. Bilateral dorsal funicular lesions alter sensorimotor behaviour in rats. Exp Neurol 205, 513-524.
Kandziora, F., Schleicher, P., Schnake, K.J., Reinhold, M., Aarabi, B., Bellabarba, C., Chapman, J., Dvorak, M., Fehlings, M., Grossman, R., Kepler, C.K., Oner, C., Shanmuganathan, R., Vialle, L.R., Vaccaro, A.R., Spinal Cord, I., Vertebral Fractures, A.K.F., 2016. [The AOSpine Classification of Thoraco-Lumbar Spine Injuries]. Z Orthop Unfall 154, 35-42.
Kang, E.S., Ha, K.Y., Kim, Y.H., 2012. Fate of transplanted bone marrow derived mesenchymal stem cells following spinal cord injury in rats by transplantation routes. J Korean Med Sci 27, 586-593.
Kapitza, S., Zorner, B., Weinmann, O., Bolliger, M., Filli, L., Dietz, V., Schwab, M.E., 2012. Tail spasms in rat spinal cord injury: changes in interneuronal connectivity. Exp Neurol 236, 179-189.
Karimi-Abdolrezaee, S., Billakanti, R., 2012. Reactive astrogliosis after spinal cord injury-beneficial and detrimental effects. Mol Neurobiol 46, 251-264.
Karimi-Abdolrezaee, S., Eftekharpour, E., Wang, J., Schut, D., Fehlings, M.G., 2010. Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord. J Neurosci 30, 1657-1676.
Kasemeier-Kulesa, J.C., Kulesa, P.M., Lefcort, F., 2005. Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. Development 132, 235-245.
Keirstead, H.S., Hasan, S.J., Muir, G.D., Steeves, J.D., 1992. Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. Proc Natl Acad Sci U S A 89, 11664-11668.

Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., Steward, O., 2005. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25, 4694-4705.

Kew, J.N., Kemp, J.A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl) 179, 4-29.

Keyvan-Fouladi, N., Raisman, G., Li, Y., 2003. Functional repair of the corticospinal tract by delayed transplantation of olfactory ensheathing cells in adult rats. J Neurosci 23, 9428-9434.

Khankan, R.R., Griffis, K.G., Haggerty-Skeans, J.R., Zhong, H., Roy, R.R., Edgerton, V.R., Phelps, P.E., 2016. Olfactory Ensheathing Cell Transplantation after a Complete Spinal Cord Transection Mediates Neuroprotective and Immunomodulatory Mechanisms to Facilitate Regeneration. J Neurosci 36, 6269-6286.

Khazaei, M., Ahuja, C.S., Fehlings, M.G., 2016. Induced Pluripotent Stem Cells for Traumatic Spinal Cord Injury. Front Cell Dev Biol 4, 152.

Kim, M., Kim, K.H., Song, S.U., Yi, T.G., Yoon, S.H., Park, S.R., Choi, B.H., 2017. Transplantation of human bone marrow-derived clonal mesenchymal stem cells reduces fibrotic scar formation in a rat spinal cord injury model. J Tissue Eng Regen Med.

Kjell, J., Olson, L., 2016. Rat models of spinal cord injury: from pathology to potential therapies. Dis Model Mech 9, 1125-1137.

Klein, S., Svendsen, C.N., 2005. Stem cells in the injured spinal cord: reducing the pain and increasing the gain. Nat Neurosci 8, 259-260.

Kojima, A., Tator, C.H., 2002. Intrathecal administration of epidermal growth factor and fibroblast growth factor 2 promotes ependymal proliferation and functional recovery after spinal cord injury in adult rats. J Neurotrauma 19, 223-238.

Kriegstein, A., Alvarez-Buylla, A., 2009. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32, 149-184.
Kumar, A., D'Souza, S.S., Moskvin, O.V., Toh, H., Wang, B., Zhang, J., Swanson, S., Guo, L.W., Thomson, J.A., Slukvin, II, 2017. Specification and Diversification of Pericytes and Smooth Muscle Cells from Mesenchymoangioblasts. Cell Rep 19, 1902-1916.
Kuroda, T., Yasuda, S., Sato, Y., 2013. Tumorigenicity studies for human pluripotent stem cell-derived products. Biol Pharm Bull 36, 189-192.
Kwok, J.C., Warren, P., Fawcett, J.W., 2012. Chondroitin sulfate: a key molecule in the brain matrix. Int J Biochem Cell Biol 44, 582-586.
Lane, M.A., Fuller, D.D., White, T.E., Reier, P.J., 2008. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. Trends Neurosci 31, 538-547.
Lange, S., Gogel, S., Leung, K.Y., Vernay, B., Nicholas, A.P., Causey, C.P., Thompson, P.R., Greene, N.D., Ferretti, P., 2011. Protein deiminases: new players in the developmentally regulated loss of neural regenerative ability. Dev Biol 355, 205-214.
Le Douarin, N., 1973. A biological cell labeling technique and its use in expermental embryology. Dev Biol 30, 217-222.
Le Douarin, N., Teillet, M.A., 1973. [Studies on the determination of neural crest-cell migration]. C R Acad Sci Hebd Seances Acad Sci D 277, 1929-1932.
Le Douarin, N.M., Smith, J., 1988. Development of the peripheral nervous system from the neural crest. Annu Rev Cell Biol 4, 375-404.
Le Lievre, C.S., Le Douarin, N.M., 1975. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. J Embryol Exp Morphol 34, 125-154.
Lee, G., Kim, H., Elkabetz, Y., Al Shamy, G., Panagiotakos, G., Barberi, T., Tabar, V., Studer, L., 2007. Isolation and directed differentiation of neural

crest stem cells derived from human embryonic stem cells. Nat Biotechnol 25, 1468-1475.
Lee, H., McKeon, R.J., Bellamkonda, R.V., 2010. Sustained delivery of thermostabilized chABC enhances axonal sprouting and functional recovery after spinal cord injury. Proc Natl Acad Sci U S A 107, 3340-3345.
Lee, J.H., Jones, C.F., Okon, E.B., Anderson, L., Tigchelaar, S., Kooner, P., Godbey, T., Chua, B., Gray, G., Hildebrandt, R., Cripton, P., Tetzlaff, W., Kwon, B.K., 2013. A novel porcine model of traumatic thoracic spinal cord injury. J Neurotrauma 30, 142-159.
Lee, S.H., Kim, Y., Rhew, D., Kuk, M., Kim, M., Kim, W.H., Kweon, O.K., 2015. Effect of the combination of mesenchymal stromal cells and chondroitinase ABC on chronic spinal cord injury. Cytotherapy 17, 1374-1383.
Lee, S.M., Yune, T.Y., Kim, S.J., Park, D.W., Lee, Y.K., Kim, Y.C., Oh, Y.J., Markelonis, G.J., Oh, T.H., 2003. Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat. J Neurotrauma 20, 1017-1027.
Leonard, A.V., Menendez, J.Y., Pat, B.M., Hadley, M.N., Floyd, C.L., 2017. Localization of the corticospinal tract within the porcine spinal cord: Implications for experimental modeling of traumatic spinal cord injury. Neurosci Lett 648, 1-7.
Lepore, A.C., Fischer, I., 2005. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. Exp Neurol 194, 230-242.
Levi, A.D., Okonkwo, D.O., Park, P., Jenkins, A.L., 3rd, Kurpad, S.N., Parr, A.M., Ganju, A., Aarabi, B., Kim, D., Casha, S., Fehlings, M.G., Harrop, J.S., Anderson, K.D., Gage, A., Hsieh, J., Huhn, S., Curt, A., Guzman, R., 2017. Emerging Safety of Intramedullary Transplantation of Human Neural Stem Cells in Chronic Cervical and Thoracic Spinal Cord Injury. Neurosurgery.

Levy, S., Benhamou, M., Naaman, C., Rainville, P., Callot, V., Cohen-Adad, J., 2015. White matter atlas of the human spinal cord with estimation of partial volume effect. Neuroimage 119, 262-271.

Li, H., Ham, T.R., Neill, N., Farrag, M., Mohrman, A.E., Koenig, A.M., Leipzig, N.D., 2016a. A Hydrogel Bridge Incorporating Immobilized Growth Factors and Neural Stem/Progenitor Cells to Treat Spinal Cord Injury. Adv Healthc Mater 5, 802-812.

Li, L., Lundkvist, A., Andersson, D., Wilhelmsson, U., Nagai, N., Pardo, A.C., Nodin, C., Stahlberg, A., Aprico, K., Larsson, K., Yabe, T., Moons, L., Fotheringham, A., Davies, I., Carmeliet, P., Schwartz, J.P., Pekna, M., Kubista, M., Blomstrand, F., Maragakis, N., Nilsson, M., Pekny, M., 2008. Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab 28, 468-481.

Li, W., Huang, L., Zeng, J., Lin, W., Li, K., Sun, J., Huang, W., Chen, J., Wang, G., Ke, Q., Duan, J., Lai, X., Chen, R., Liu, M., Liu, Y., Wang, T., Yang, X., Chen, Y., Xia, H., Xiang, A.P., 2016b. Characterization and transplantation of enteric neural crest cells from human induced pluripotent stem cells. Mol Psychiatry.

Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., Fouad, K., Mir, A., Rausch, M., Kindler, D., Hamers, F.P., Schwab, M.E., 2005. Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. Ann Neurol 58, 706-719.

Lim, S.W., Shiue, Y.L., Ho, C.H., Yu, S.C., Kao, P.H., Wang, J.J., Kuo, J.R., 2017. Anxiety and Depression in Patients with Traumatic Spinal Cord Injury: A Nationwide Population-Based Cohort Study. PLoS One 12, e0169623.

Lindvall, O., Bjorklund, A., 2004. Cell therapy in Parkinson's disease. NeuroRx 1, 382-393.

Litvan, I., Halliday, G., Hallett, M., Goetz, C.G., Rocca, W., Duyckaerts, C., Ben-Shlomo, Y., Dickson, D.W., Lang, A.E., Chesselet, M.F., Langston, W.J., Di Monte, D.A., Gasser, T., Hagg, T., Hardy, J., Jenner, P., Melamed, E., Myers, R.H., Parker, D., Jr., Price, D.L., 2007. The etiopathogenesis of Parkinson disease and suggestions for future research. Part I. J Neuropathol Exp Neurol 66, 251-257.

Liu, Z., Xin, H., Chopp, M., 2014. Reactive astrocytes promote axonal remodeling and neurological recovery after stroke. Neural Regen Res 9, 1874-1875.

Lo, W.C., Chiou, J.F., Gelovani, J.G., Cheong, M.L., Lee, C.M., Liu, H.Y., Wu, C.H., Wang, M.F., Lin, C.T., Deng, W.P., 2009. Transplantation of embryonic fibroblasts treated with platelet-rich plasma induces osteogenesis in SAMP8 mice monitored by molecular imaging. J Nucl Med 50, 765-773.

Lowenthal, J.W., Connick, T.E., McWaters, P.G., York, J.J., 1994. Development of T cell immune responsiveness in the chicken. Immunol Cell Biol 72, 115-122.

Lowery, L.A., Van Vactor, D., 2009. The trip of the tip: understanding the growth cone machinery. Nat Rev Mol Cell Biol 10, 332-343.

Loy, D.N., Crawford, C.H., Darnall, J.B., Burke, D.A., Onifer, S.M., Whittemore, S.R., 2002. Temporal progression of angiogenesis and basal lamina deposition after contusive spinal cord injury in the adult rat. J Comp Neurol 445, 308-324.

Loyd, D.R., Henry, M.A., Hargreaves, K.M., 2013. Serotonergic neuromodulation of peripheral nociceptors. Semin Cell Dev Biol 24, 51-57.

Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H., 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol 181, 115-129.

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., Brock, J., Blesch, A., Rosenzweig, E.S., Havton, L.A., Zheng, B., Conner, J.M., Marsala, M., Tuszynski, M.H., 2012. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150, 1264-1273.

Lu, P., Woodruff, G., Wang, Y., Graham, L., Hunt, M., Wu, D., Boehle, E., Ahmad, R., Poplawski, G., Brock, J., Goldstein, L.S., Tuszynski, M.H., 2014. Long-distance axonal growth from human induced pluripotent stem cells after spinal cord injury. Neuron 83, 789-796.

Lu, P., Yang, H., Jones, L.L., Filbin, M.T., Tuszynski, M.H., 2004. Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. J Neurosci 24, 6402-6409.

Lukovic, D., Stojkovic, M., Moreno-Manzano, V., Bhattacharya, S.S., Erceg, S., 2014. Perspectives and future directions of human pluripotent stem cell-based therapies: lessons from Geron's clinical trial for spinal cord injury. Stem Cells Dev 23, 1-4.

Lukovic, D., Stojkovic, M., Moreno-Manzano, V., Jendelova, P., Sykova, E., Bhattacharya, S.S., Erceg, S., 2015. Concise review: reactive astrocytes and stem cells in spinal cord injury: good guys or bad guys? Stem Cells 33, 1036-1041.

Lund, R.J., Narva, E., Lahesmaa, R., 2012. Genetic and epigenetic stability of human pluripotent stem cells. Nat Rev Genet 13, 732-744.

Lyczek, A., Arnold, A., Zhang, J., Campanelli, J.T., Janowski, M., Bulte, J.W., Walczak, P., 2017. Transplanted human glial-restricted progenitors can rescue the survival of dysmyelinated mice independent of the production of mature, compact myelin. Exp Neurol 291, 74-86.

Ma, M., Basso, D.M., Walters, P., Stokes, B.T., Jakeman, L.B., 2001. Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. Exp Neurol 169, 239-254.

Macklin WB, CL, W., 1985. Appearance of myelin proteins during development in the chick central nervous system. Developmental Neuroscience 7, 170-178.
Macklin, W.B., Weill, C.L., 1985. Appearance of myelin proteins during development in the chick central nervous system. Dev Neurosci 7, 170-178.
Martinez-Hernandez, A., Bell, K.P., Norenberg, M.D., 1977. Glutamine synthetase: glial localization in brain. Science 195, 1356-1358.
Matsuyama, Y., Sato, K., Kamiya, M., Yano, J., Iwata, H., Isobe, K., 1998. Nitric oxide: a possible etiologic factor in spinal cord cavitation. J Spinal Disord 11, 248-252.
Mautes, A.E., Weinzierl, M.R., Donovan, F., Noble, L.J., 2000. Vascular events after spinal cord injury: contribution to secondary pathogenesis. Phys Ther 80, 673-687.
McCall, J., Weidner, N., Blesch, A., 2012. Neurotrophic factors in combinatorial approaches for spinal cord regeneration. Cell Tissue Res 349, 27-37.
McCann, C.J., Cooper, J.E., Natarajan, D., Jevans, B., Burnett, L.E., Burns, A.J., Thapar, N., 2017. Transplantation of enteric nervous system stem cells rescues nitric oxide synthase deficient mouse colon. Nat Commun 8, 15937.
McConalogue, K., Furness, J.B., 1994. Gastrointestinal neurotransmitters. Baillieres Clin Endocrinol Metab 8, 51-76.
McConalogue, K., Low, A.M., Williamson, S., Bornstein, J.C., Furness, J.B., 1994. Calretinin-immunoreactive neurons and their projections in the guinea-pig colon. Cell Tissue Res 276, 359-365.
McConnell, J.A., Sechrist, J.W., 1980. Identification of early neurons in the brainstem and spinal cord: I. An autoradiographic study in the chick. J Comp Neurol 192, 769-783.

McDermott, K.W., Barry, D.S., McMahon, S.S., 2005. Role of radial glia in cytogenesis, patterning and boundary formation in the developing spinal cord. J Anat 207, 241-250.
McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I., Choi, D.W., 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 5, 1410-1412.
McDonald, J.W., Sadowsky, C., 2002. Spinal-cord injury. Lancet 359, 417-425.
McGee, A.W., Strittmatter, S.M., 2003. The Nogo-66 receptor: focusing myelin inhibition of axon regeneration. Trends Neurosci 26, 193-198.
McGrew, M.J., Sherman, A., Ellard, F.M., Lillico, S.G., Gilhooley, H.J., Kingsman, A.J., Mitrophanous, K.A., Sang, H., 2004. Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep 5, 728-733.
McKeon, R.J., Hoke, A., Silver, J., 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136, 32-43.
McKeon, R.J., Schreiber, R.C., Rudge, J.S., Silver, J., 1991. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci 11, 3398-3411.
McLennan, R., Schumacher, L.J., Morrison, J.A., Teddy, J.M., Ridenour, D.A., Box, A.C., Semerad, C.L., Li, H., McDowell, W., Kay, D., Maini, P.K., Baker, R.E., Kulesa, P.M., 2015. Neural crest migration is driven by a few trailblazer cells with a unique molecular signature narrowly confined to the invasive front. Development 142, 2014-2025.
Mendonca, M.V., Larocca, T.F., de Freitas Souza, B.S., Villarreal, C.F., Silva, L.F., Matos, A.C., Novaes, M.A., Bahia, C.M., de Oliveira Melo Martinez,

A.C., Kaneto, C.M., Furtado, S.B., Sampaio, G.P., Soares, M.B., dos Santos, R.R., 2014. Safety and neurological assessments after autologous transplantation of bone marrow mesenchymal stem cells in subjects with chronic spinal cord injury. Stem Cell Res Ther 5, 126.

Meng, F., Modo, M., Badylak, S.F., 2014. Biologic scaffold for CNS repair. Regen Med 9, 367-383.

Merkler, D., Metz, G.A., Raineteau, O., Dietz, V., Schwab, M.E., Fouad, K., 2001. Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. J Neurosci 21, 3665-3673.

Metzger, M., Bareiss, P.M., Danker, T., Wagner, S., Hennenlotter, J., Guenther, E., Obermayr, F., Stenzl, A., Koenigsrainer, A., Skutella, T., Just, L., 2009a. Expansion and differentiation of neural progenitors derived from the human adult enteric nervous system. Gastroenterology 137, 2063-2073 e2064.

Metzger, M., Caldwell, C., Barlow, A.J., Burns, A.J., Thapar, N., 2009b. Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology 136, 2214-2225 e2211-2213.

Miao, Q.L., Ye, Q., Zhang, X.H., 2014. Perineuronal net, CSPG receptor and their regulation of neural plasticity. Sheng Li Xue Bao 66, 387-397.

Mietto, B., Costa, R., de Lima, S., ST., F., AMB., M., 2011. Wallerian Degeneration in Injury and Diseases: Concepts and Prevention, Advanced Understanding of Neurodegenerative Diseases. ISBN.

Mietto, B.S., Mostacada, K., Martinez, A.M., 2015. Neurotrauma and inflammation: CNS and PNS responses. Mediators Inflamm 2015, 251204.

Mikkelsen, H.B., Rumessen, J.J., 1992. Characterization of macrophage-like cells in the external layers of human small and large intestine. Cell Tissue Res 270, 273-279.
Mikkelsen, H.B., Thuneberg, L., Rumessen, J.J., Thorball, N., 1985. Macrophage-like cells in the muscularis externa of mouse small intestine. Anat Rec 213, 77-86.
Miletich, I., Sharpe, P.T., 2004. Neural crest contribution to mammalian tooth formation. Birth Defects Res C Embryo Today 72, 200-212.
Mitsui, T., Shumsky, J.S., Lepore, A.C., Murray, M., Fischer, I., 2005. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. J Neurosci 25, 9624-9636.
Moeendarbary, E., Weber, I.P., Sheridan, G.K., Koser, D.E., Soleman, S., Haenzi, B., Bradbury, E.J., Fawcett, J., Franze, K., 2017. The soft mechanical signature of glial scars in the central nervous system. Nat Commun 8, 14787.
Moon, L.D., Asher, R.A., Rhodes, K.E., Fawcett, J.W., 2001. Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. Nat Neurosci 4, 465-466.
Moreno, A., Zidarov, D., Raju, C., Boruff, J., Ahmed, S., 2017. Integrating the perspectives of individuals with spinal cord injuries, their family caregivers and healthcare professionals from the time of rehabilitation admission to community reintegration: protocol for a scoping study on SCI needs. BMJ Open 7, e014331.
Morgenstern DA, Asher RA, JW, F., 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 137, 313-332.
Morgenstern, D.A., Asher, R.A., Fawcett, J.W., 2002. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 137, 313-332.

Morris, R., Tosolini, A.P., Goldstein, J.D., Whishaw, I.Q., 2011. Impaired arpeggio movement in skilled reaching by rubrospinal tract lesions in the rat: a behavioral/anatomical fractionation. J Neurotrauma 28, 2439-2451.
Mothe, A.J., Kulbatski, I., Parr, A., Mohareb, M., Tator, C.H., 2008. Adult spinal cord stem/progenitor cells transplanted as neurospheres preferentially differentiate into oligodendrocytes in the adult rat spinal cord. Cell Transplant 17, 735-751.
Mothe, A.J., Tator, C.H., 2012. Advances in stem cell therapy for spinal cord injury. J Clin Invest 122, 3824-3834.
Moticka, E., 1975. Development of immunological competenence in chickens. Amer. Zool. 15, 135-146.
Muir, E.M., Fyfe, I., Gardiner, S., Li, L., Warren, P., Fawcett, J.W., Keynes, R.J., Rogers, J.H., 2010. Modification of N-glycosylation sites allows secretion of bacterial chondroitinase ABC from mammalian cells. J Biotechnol 145, 103-110.
Mulak, A., Bonaz, B., 2015. Brain-gut-microbiota axis in Parkinson's disease. World J Gastroenterol 21, 10609-10620.
Murphy, J.B., 1914. Factors of Resistance to Heteroplastic Tissue-Grafting : Studies in Tissue Specificity. Iii. J Exp Med 19, 513-522.
Murray, K.C., Nakae, A., Stephens, M.J., Rank, M., D'Amico, J., Harvey, P.J., Li, X., Harris, R.L., Ballou, E.W., Anelli, R., Heckman, C.J., Mashimo, T., Vavrek, R., Sanelli, L., Gorassini, M.A., Bennett, D.J., Fouad, K., 2010. Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat Med 16, 694-700.
Myslinski, N.R., Randic, M., 1977. Responses of identified spinal neurones to acetylcholine applied by micro-electrophoresis. J Physiol 269, 195-219.
Nakamura, M., Okano, H., 2013. Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells. Cell Res 23, 70-80.

Nas, K., Yazmalar, L., Sah, V., Aydin, A., Ones, K., 2015. Rehabilitation of spinal cord injuries. World J Orthop 6, 8-16.

Natarajan, D., Cooper, J., Choudhury, S., Delalande, J.M., McCann, C., Howe, S.J., Thapar, N., Burns, A.J., 2014. Lentiviral labeling of mouse and human enteric nervous system stem cells for regenerative medicine studies. Neurogastroenterol Motil 26, 1513-1518.

Natarajan, D., Grigoriou, M., Marcos-Gutierrez, C., Atkins, C., Pachnis, V., 1999. Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture. Development 126, 157-168.

National Spinal Cord InjuryStatistical, C., 2014. Spinal Cord Injury Facts and Figures at a Glance. J Spinal Cord Med 37, 355-356.

Navlakha, S., Suhan, J., Barth, A.L., Bar-Joseph, Z., 2013. A high-throughput framework to detect synapses in electron microscopy images. Bioinformatics 29, i9-17.

Negash, T., al-Garib, S.O., Gruys, E., 2004. Comparison of in ovo and post-hatch vaccination with particular reference to infectious bursal disease. A review. Vet Q 26, 76-87.

Newbern, J.M., 2015. Molecular control of the neural crest and peripheral nervous system development. Curr Top Dev Biol 111, 201-231.

Nezami, B.G., Srinivasan, S., 2010. Enteric nervous system in the small intestine: pathophysiology and clinical implications. Curr Gastroenterol Rep 12, 358-365.

Nguyen, H., O'Barr, T., Anderson, A., 2007. Polymorphonuclear leukocytes promote neurotoxicity throughrelease of matrix metalloproteinases, reactive oxygen species,and TNF-a. Journal of Neurochemistry 102, 900-912.

Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K., Keirstead, H.S., 2005. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49, 385-396.
Noble, L.J., Wrathall, J.R., 1989. Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. Exp Neurol 103, 34-40.
Nobunaga, A.I., Go, B.K., Karunas, R.B., 1999. Recent demographic and injury trends in people served by the Model Spinal Cord Injury Care Systems. Arch Phys Med Rehabil 80, 1372-1382.
Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., Kriegstein, A.R., 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7, 136-144.
Novikova, L.N., Brohlin, M., Kingham, P.J., Novikov, L.N., Wiberg, M., 2011. Neuroprotective and growth-promoting effects of bone marrow stromal cells after cervical spinal cord injury in adult rats. Cytotherapy 13, 873-887.
O'Neill, P., Whalley, K., Ferretti, P., 2004. Nogo and Nogo-66 receptor in human and chick: implications for development and regeneration. Dev Dyn 231, 109-121.
Ogawa, Y., Sawamoto, K., Miyata, T., Miyao, S., Watanabe, M., Nakamura, M., Bregman, B.S., Koike, M., Uchiyama, Y., Toyama, Y., Okano, H., 2002. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res 69, 925-933.
Ohtake, Y., Li, S., 2015. Molecular mechanisms of scar-sourced axon growth inhibitors. Brain Res 1619, 22-35.
Oliveri, R.S., Bello, S., Biering-Sorensen, F., 2014. Mesenchymal stem cells improve locomotor recovery in traumatic spinal cord injury: systematic review with meta-analyses of rat models. Neurobiol Dis 62, 338-353.

Onifer, S.M., Rabchevsky, A.G., Scheff, S.W., 2007. Rat models of traumatic spinal cord injury to assess motor recovery. ILAR J 48, 385-395.
Pai, Y.J., Abdullah, N.L., Mohd-Zin, S.W., Mohammed, R.S., Rolo, A., Greene, N.D., Abdul-Aziz, N.M., Copp, A.J., 2012. Epithelial fusion during neural tube morphogenesis. Birth Defects Res A Clin Mol Teratol 94, 817-823.
Palimaru, A., Cunningham, W.E., Dillistone, M., Vargas-Bustamante, A., Liu, H., Hays, R.D., 2017. A comparison of perceptions of quality of life among adults with spinal cord injury in the United States versus the United Kingdom. Qual Life Res.
Pan, H.C., Cheng, F.C., Lai, S.Z., Yang, D.Y., Wang, Y.C., Lee, M.S., 2008. Enhanced regeneration in spinal cord injury by concomitant treatment with granulocyte colony-stimulating factor and neuronal stem cells. J Clin Neurosci 15, 656-664.
Pan-Montojo, F., Anichtchik, O., Dening, Y., Knels, L., Pursche, S., Jung, R., Jackson, S., Gille, G., Spillantini, M.G., Reichmann, H., Funk, R.H., 2010. Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. PLoS One 5, e8762.
Panayiotou, E., Malas, S., 2013. Adult spinal cord ependymal layer: a promising pool of quiescent stem cells to treat spinal cord injury. Front Physiol 4, 340.
Pander, C., 1817. Beiträge zur Entwickelungsgeschichte des Hühnchens im Eie [Contributions to the Developmental History of the Chicken in the Egg].
Paridaen, J.T., Huttner, W.B., 2014. Neurogenesis during development of the vertebrate central nervous system. EMBO Rep 15, 351-364.
Park, E., Velumian, A.A., Fehlings, M.G., 2004. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J Neurotrauma 21, 754-774.

Park, J.H., Kim, D.Y., Sung, I.Y., Choi, G.H., Jeon, M.H., Kim, K.K., Jeon, S.R., 2012. Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. Neurosurgery 70, 1238-1247; discussion 1247.

Parr, A.M., Kulbatski, I., Wang, X.H., Keating, A., Tator, C.H., 2008. Fate of transplanted adult neural stem/progenitor cells and bone marrow-derived mesenchymal stromal cells in the injured adult rat spinal cord and impact on functional recovery. Surg Neurol 70, 600-607; discussion 607.

Perea, G., Navarrete, M., Araque, A., 2009. Tripartite synapses: astrocytes process and control synaptic information. Trends Neurosci 32, 421-431.

Perris, R., Johansson, S., 1990. Inhibition of neural crest cell migration by aggregating chondroitin sulfate proteoglycans is mediated by their hyaluronan-binding region. Dev Biol 137, 1-12.

Piltti, K.M., Avakian, S.N., Funes, G.M., Hu, A., Uchida, N., Anderson, A.J., Cummings, B.J., 2015. Transplantation dose alters the dynamics of human neural stem cell engraftment, proliferation and migration after spinal cord injury. Stem Cell Res 15, 341-353.

Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J.W., Maffei, L., 2002. Reactivation of ocular dominance plasticity in the adult visual cortex. Science 298, 1248-1251.

Plemel, J.R., Keough, M.B., Duncan, G.J., Sparling, J.S., Yong, V.W., Stys, P.K., Tetzlaff, W., 2014. Remyelination after spinal cord injury: is it a target for repair? Prog Neurobiol 117, 54-72.

Popovich, P.G., 2012. Building bridges for spinal cord repair. Cell 150, 1105-1106.

Poser, S.W., Androutsellis-Theotokis, A., 2013. Growing neural stem cells from conventional and nonconventional regions of the adult rodent brain. J Vis Exp, e50880.

Prabhakar, V., Capila, I., Bosques, C.J., Pojasek, K., Sasisekharan, R., 2005a. Chondroitinase ABC I from Proteus vulgaris: cloning, recombinant expression and active site identification. Biochem J 386, 103-112.

Prabhakar, V., Raman, R., Capila, I., Bosques, C.J., Pojasek, K., Sasisekharan, R., 2005b. Biochemical characterization of the chondroitinase ABC I active site. Biochem J 390, 395-405.

Prasongchean, W., Bagni, M., Calzarossa, C., De Coppi, P., Ferretti, P., 2012. Amniotic fluid stem cells increase embryo survival following injury. Stem Cells Dev 21, 675-688.

Priest, C.A., Manley, N.C., Denham, J., Wirth, E.D., 3rd, Lebkowski, J.S., 2015. Preclinical safety of human embryonic stem cell-derived oligodendrocyte progenitors supporting clinical trials in spinal cord injury. Regen Med 10, 939-958.

Qu, J., Zhang, H., 2017. Roles of Mesenchymal Stem Cells in Spinal Cord Injury. Stem Cells International 2017.

Rahimi-Movaghar, V., 2009. Clinical trials for the treatment of spinal cord injury: cervical and lumbar enlargements versus thoracic area. Brain 132, e115; author reply e116.

Ramon-Cueto, A., Cordero, M.I., Santos-Benito, F.F., Avila, J., 2000. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25, 425-435.

Rasouli, A., Bhatia, N., Dinh, P., Cahill, K., Suryadevara, S., Gupta, R., 2009. Resection of glial scar following spinal cord injury. J Orthop Res 27, 931-936.

Reier, P.J., Houle, J.D., 1988. The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair. Adv Neurol 47, 87-138.

Renault-Mihara, F., Okada, S., Shibata, S., Nakamura, M., Toyama, Y., Okano, H., 2008. Spinal cord injury: emerging beneficial role of reactive astrocytes' migration. Int J Biochem Cell Biol 40, 1649-1653.

Richardson, P.M., McGuinness, U.M., Aguayo, A.J., 1980. Axons from CNS neurons regenerate into PNS grafts. Nature 284, 264-265.
Roberts, E., Frankel, S., 1950. gamma-Aminobutyric acid in brain: its formation from glutamic acid. J Biol Chem 187, 55-63.
Rolls, A., Avidan, H., Cahalon, L., Schori, H., Bakalash, S., Litvak, V., Lev, S., Lider, O., Schwartz, M., 2004. A disaccharide derived from chondroitin sulphate proteoglycan promotes central nervous system repair in rats and mice. Eur J Neurosci 20, 1973-1983.
Rolls, A., Shechter, R., London, A., Segev, Y., Jacob-Hirsch, J., Amariglio, N., Rechavi, G., Schwartz, M., 2008. Two faces of chondroitin sulfate proteoglycan in spinal cord repair: a role in microglia/macrophage activation. PLoS Med 5, e171.
Rolls, A., Shechter, R., Schwartz, M., 2009. The bright side of the glial scar in CNS repair. Nat Rev Neurosci 10, 235-241.
Rouanet, C., Reges, D., Rocha, E., Gagliardi, V., Silva, G.S., 2017. Traumatic spinal cord injury: current concepts and treatment update. Arq Neuropsiquiatr 75, 387-393.
Rowitch, D.H., 2004. Glial specification in the vertebrate neural tube. Nat Rev Neurosci 5, 409-419.
Ruan, X., Luo, J.J., 2017. Suicide before and after spinal cord injury. J Spinal Cord Med 40, 280-281.
Ruff, R.L., McKerracher, L., Selzer, M.E., 2008. Repair and neurorehabilitation strategies for spinal cord injury. Ann N Y Acad Sci 1142, 1-20.
Ruhl, A., 2006. Glial regulation of neuronal plasticity in the gut: implications for clinicians. Gut 55, 600-602.
Ruzicka, J., Machova-Urdzikova, L., Gillick, J., Amemori, T., Romanyuk, N., Karova, K., Zaviskova, K., Dubisova, J., Kubinova, S., Murali, R., Sykova, E., Jhanwar-Uniyal, M., Jendelova, P., 2017. A Comparative Study of Three

Different Types of Stem Cells for Treatment of Rat Spinal Cord Injury. Cell Transplant 26, 585-603.
Ryu, H.H., Lim, J.H., Byeon, Y.E., Park, J.R., Seo, M.S., Lee, Y.W., Kim, W.H., Kang, K.S., Kweon, O.K., 2009. Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. J Vet Sci 10, 273-284.
Sarveazad, A., Babahajian, A., Bakhtiari, M., Soleimani, M., Behnam, B., Yari, A., Akbari, A., Yousefifard, M., Janzadeh, A., Amini, N., Agah, S., Fallah, A., Joghataei, M.T., 2017. The combined application of human adipose derived stem cells and Chondroitinase ABC in treatment of a spinal cord injury model. Neuropeptides 61, 39-47.
Sarveazad, A., Bakhtiari, M., Babahajian, A., Janzade, A., Fallah, A., Moradi, F., Soleimani, M., Younesi, M., Goudarzi, F., Mohammad Taghi, J., 2014. Comparison of human adipose-derived stem cells and chondroitinase ABC transplantation on locomotor recovery in the contusion model of spinal cord injury in rats. Iran J Basic Med Sci 17, 685-693.
Satti, H.S., Waheed, A., Ahmed, P., Ahmed, K., Akram, Z., Aziz, T., Satti, T.M., Shahbaz, N., Khan, M.A., Malik, S.A., 2016. Autologous mesenchymal stromal cell transplantation for spinal cord injury: A Phase I pilot study. Cytotherapy 18, 518-522.
Satzer, D., Miller, C., Maxon, J., Voth, J., DiBartolomeo, C., Mahoney, R., Dutton, J.R., Low, W.C., Parr, A.M., 2015. T cell deficiency in spinal cord injury: altered locomotor recovery and whole-genome transcriptional analysis. BMC Neurosci 16, 74.
Sauer, F., 1935. The cellular structure of the neural tube. The Journal of Comparative Neurology 63, 13-23.
Saunders, J.W., Jr., 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J Exp Zool 108, 363-403.

Schmidt, B.J., Jordan, L.M., 2000. The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. Brain Res Bull 53, 689-710.
Schomberg, D.T., Miranpuri, G.S., Chopra, A., Patel, K., Meudt, J.J., Tellez, A., Resnick, D.K., Shanmuganayagam, D., 2017. Translational Relevance of Swine Models of Spinal Cord Injury. J Neurotrauma 34, 541-551.
Scuteri, A., Miloso, M., Foudah, D., Orciani, M., Cavaletti, G., Tredici, G., 2011. Mesenchymal stem cells neuronal differentiation ability: a real perspective for nervous system repair? Curr Stem Cell Res Ther 6, 82-92.
Sekiguchi, K.J., Shekhtmeyster, P., Merten, K., Arena, A., Cook, D., Hoffman, E., Ngo, A., Nimmerjahn, A., 2016. Imaging large-scale cellular activity in spinal cord of freely behaving mice. Nat Commun 7, 11450.
Seto, F., 1981. Early development of the avian immune system. Poult Sci 60, 1981-1995.
Sezer, N., Akkus, S., Ugurlu, F.G., 2015. Chronic complications of spinal cord injury. World J Orthop 6, 24-33.
Sharif-Alhoseini, M., Khormali, M., Rezaei, M., Safdarian, M., Hajighadery, A., Khalatbari, M.M., Safdarian, M., Meknatkhah, S., Rezvan, M., Chalangari, M., Derakhshan, P., Rahimi-Movaghar, V., 2017. Animal models of spinal cord injury: a systematic review. Spinal Cord.
Shepherd, G.M., Raastad, M., 2003. Axonal varicosity distributions along parallel fibers: a new angle on a cerebellar circuit. Cerebellum 2, 110-113.
Sherwin, E., Rea, K., Dinan, T.G., Cryan, J.F., 2016. A gut (microbiome) feeling about the brain. Curr Opin Gastroenterol 32, 96-102.
Shimizu, I., Oppenheim, R.W., O'Brien, M., Shneiderman, A., 1990. Anatomical and functional recovery following spinal cord transection in the chick embryo. J Neurobiol 21, 918-937.

Shyamala, K., Yanduri, S., Girish, H.C., Murgod, S., 2015. Neural crest: The fourth germ layer. J Oral Maxillofac Pathol 19, 221-229.
Siebert, J.R., Conta Steencken, A., Osterhout, D.J., 2014. Chondroitin sulfate proteoglycans in the nervous system: inhibitors to repair. Biomed Res Int 2014, 845323.
Silva, M., Daheron, L., Hurley, H., Bure, K., Barker, R., Carr, A.J., Williams, D., Kim, H.W., French, A., Coffey, P.J., Cooper-White, J.J., Reeve, B., Rao, M., Snyder, E.Y., Ng, K.S., Mead, B.E., Smith, J.A., Karp, J.M., Brindley, D.A., Wall, I., 2015. Generating iPSCs: translating cell reprogramming science into scalable and robust biomanufacturing strategies. Cell Stem Cell 16, 13-17.
Silver, D.J., Silver, J., 2014. Contributions of chondroitin sulfate proteoglycans to neurodevelopment, injury, and cancer. Curr Opin Neurobiol 27, 171-178.
Silver, J., Miller, J.H., 2004. Regeneration beyond the glial scar. Nat Rev Neurosci 5, 146-156.
Simpson, L.A., Eng, J.J., Hsieh, J.T., Wolfe, D.L., Spinal Cord Injury Rehabilitation Evidence Scire Research, T., 2012. The health and life priorities of individuals with spinal cord injury: a systematic review. J Neurotrauma 29, 1548-1555.
Slaughter, B.V., Khurshid, S.S., Fisher, O.Z., Khademhosseini, A., Peppas, N.A., 2009. Hydrogels in regenerative medicine. Adv Mater 21, 3307-3329.
Smith, R.R., Shum-Siu, A., Baltzley, R., Bunger, M., Baldini, A., Burke, D.A., Magnuson, D.S., 2006. Effects of swimming on functional recovery after incomplete spinal cord injury in rats. J Neurotrauma 23, 908-919.
Snow, D.M., Letourneau, P.C., 1992. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). J Neurobiol 23, 322-336.
Soden, R.J., Walsh, J., Middleton, J.W., Craven, M.L., Rutkowski, S.B., Yeo, J.D., 2000. Causes of death after spinal cord injury. Spinal Cord 38, 604-610.

Song, L.H., Pan, W., Yu, Y.H., Quarles, L.D., Zhou, H.H., Xiao, Z.S., 2006. Resveratrol prevents CsA inhibition of proliferation and osteoblastic differentiation of mouse bone marrow-derived mesenchymal stem cells through an ER/NO/cGMP pathway. Toxicol In Vitro 20, 915-922.
Soula, C., Danesin, C., Kan, P., Grob, M., Poncet, C., Cochard, P., 2001. Distinct sites of origin of oligodendrocytes and somatic motoneurons in the chick spinal cord: oligodendrocytes arise from Nkx2.2-expressing progenitors by a Shh-dependent mechanism. Development 128, 1369-1379.
Steigman, S.A., Fauza, D.O., 2007. Isolation of mesenchymal stem cells from amniotic fluid and placenta. Curr Protoc Stem Cell Biol Chapter 1, Unit 1E 2.
Stern, C.D., 2005. The chick; a great model system becomes even greater. Dev Cell 8, 9-17.
Steward, O., Willenberg, R., 2017. Rodent spinal cord injury models for studies of axon regeneration. Exp Neurol 287, 374-383.
Stichel, C.C., Hermanns, S., Luhmann, H.J., Lausberg, F., Niermann, H., D'Urso, D., Servos, G., Hartwig, H.G., Muller, H.W., 1999. Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. Eur J Neurosci 11, 632-646.
Suarez-Rodriguez, R., Belkind-Gerson, J., 2004. Cultured nestin-positive cells from postnatal mouse small bowel differentiate ex vivo into neurons, glia, and smooth muscle. Stem Cells 22, 1373-1385.
Sun, D., Jakobs, T.C., 2012. Structural remodeling of astrocytes in the injured CNS. Neuroscientist 18, 567-588.
Suzuki, H., Ahuja, C.S., Salewski, R.P., Li, L., Satkunendrarajah, K., Nagoshi, N., Shibata, S., Fehlings, M.G., 2017. Neural stem cell mediated recovery is enhanced by Chondroitinase ABC pretreatment in chronic cervical spinal cord injury. PLoS One 12, e0182339.

Sweet, S.N., Noreau, L., Leblond, J., Dumont, F.S., 2014. Understanding Quality of Life in Adults with Spinal Cord Injury Via SCI-Related Needs and Secondary Complications. Top Spinal Cord Inj Rehabil 20, 321-328.

Tabakow, P., Jarmundowicz, W., Czapiga, B., Fortuna, W., Miedzybrodzki, R., Czyz, M., Huber, J., Szarek, D., Okurowski, S., Szewczyk, P., Gorski, A., Raisman, G., 2013. Transplantation of autologous olfactory ensheathing cells in complete human spinal cord injury. Cell Transplant 22, 1591-1612.

Tabakow, P., Raisman, G., Fortuna, W., Czyz, M., Huber, J., Li, D., Szewczyk, P., Okurowski, S., Miedzybrodzki, R., Czapiga, B., Salomon, B., Halon, A., Li, Y., Lipiec, J., Kulczyk, A., Jarmundowicz, W., 2014. Functional regeneration of supraspinal connections in a patient with transected spinal cord following transplantation of bulbar olfactory ensheathing cells with peripheral nerve bridging. Cell Transplant 23, 1631-1655.

Tator, C., 1991. Review of experimental spinal cord injury with emphasison the local and systemic circulatory effects. Neuro Chirurgie 37, 291-302.

Tator, C.H., 1995. Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol 5, 407-413.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., Jeon, N.L., 2005. A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat Methods 2, 599-605.

Teillet, M.A., Kalcheim, C., Le Douarin, N.M., 1987. Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. Dev Biol 120, 329-347.

Teng, Y.D., Lavik, E.B., Qu, X., Park, K.I., Ourednik, J., Zurakowski, D., Langer, R., Snyder, E.Y., 2002. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 99, 3024-3029.

Toma, J.S., Shettar, B.C., Chipman, P.H., Pinto, D.M., Borowska, J.P., Ichida, J.K., Fawcett, J.P., Zhang, Y., Eggan, K., Rafuse, V.F., 2015. Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. J Neurosci 35, 1291-1306.
Twarog, B.M., Page, I.H., 1953. Serotonin content of some mammalian tissues and urine and a method for its determination. Am J Physiol 175, 157-161.
Uesaka, T., Nagashimada, M., Enomoto, H., 2015. Neuronal Differentiation in Schwann Cell Lineage Underlies Postnatal Neurogenesis in the Enteric Nervous System. J Neurosci 35, 9879-9888.
Ulusoy, A., Phillips, R.J., Helwig, M., Klinkenberg, M., Powley, T.L., Di Monte, D.A., 2017. Brain-to-stomach transfer of alpha-synuclein via vagal preganglionic projections. Acta Neuropathol 133, 381-393.
Vaglia, J.L., Hall, B.K., 1999. Regulation of neural crest cell populations: occurrence, distribution and underlying mechanisms. Int J Dev Biol 43, 95-110.
van Straaten, H.W., Hekking, J.W., Thors, F., Wiertz-Hoessels, E.L., Drukker, J., 1985. Induction of an additional floor plate in the neural tube. Acta Morphol Neerl Scand 23, 91-97.
Vargas, M.E., Barres, B.A., 2007. Why is Wallerian degeneration in the CNS so slow? Annu Rev Neurosci 30, 153-179.
Vialli, M., Erspamer, V., 1937. Ricerche sul secreto delle cellule enterocromaffini. IX Intorno alla natura chimica della sostanza specifica. Boll. Soc. Med.-Chir. 51, 1111–1116.
Vismara, I., Papa, S., Rossi, F., Forloni, G., Veglianese, P., 2017. Current Options for Cell Therapy in Spinal Cord Injury. Trends Mol Med.
von Joest, M., Bua Aguin, S., Li, H., 2016. Genomic stability during cellular reprogramming: Mission impossible? Mutat Res 788, 12-16.

Waddington, C.H., 1950. Processes of induction in the early development of the chick. Annee Biol 54, 711-717.
Wakabayashi, K., Takahashi, H., Takeda, S., Ohama, E., Ikuta, F., 1989. Lewy bodies in the enteric nervous system in Parkinson's disease. Arch Histol Cytol 52 Suppl, 191-194.
Wallace, A.S., Burns, A.J., 2005. Development of the enteric nervous system, smooth muscle and interstitial cells of Cajal in the human gastrointestinal tract. Cell Tissue Res 319, 367-382.
Wallach, M., Halabi, A., Pillemer, G., Sar-Shalom, O., Mencher, D., Gilad, M., Bendheim, U., Danforth, H.D., Augustine, P.C., 1992. Maternal immunization with gametocyte antigens as a means of providing protective immunity against Eimeria maxima in chickens. Infect Immun 60, 2036-2039.
Walters, E.M., Wells, K.D., Bryda, E.C., Schommer, S., Prather, R.S., 2017. Swine models, genomic tools and services to enhance our understanding of human health and diseases. Lab Anim (NY) 46, 167-172.
Wang, X., Budel, S., Baughman, K., Gould, G., Song, K., Strittmatter, S., 2009. Ibuprofen Enhances Recovery from Spinal Cord Injury by Limiting Tissue Loss and Stimulating Axonal Growth. Journal of Neurotrauma 26, 81-95.
Wang, X., Chan, A.K., Sham, M.H., Burns, A.J., Chan, W.Y., 2011. Analysis of the sacral neural crest cell contribution to the hindgut enteric nervous system in the mouse embryo. Gastroenterology 141, 992-1002 e1001-1006.
Whetstone, W.D., Hsu, J.Y., Eisenberg, M., Werb, Z., Noble-Haeusslein, L.J., 2003. Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. J Neurosci Res 74, 227-239.
Wictorin, K., Bjorklund, A., 1992. Axon outgrowth from grafts of human embryonic spinal cord in the lesioned adult rat spinal cord. Neuroreport 3, 1045-1048.

Wiese, S., Faissner, A., 2015. The role of extracellular matrix in spinal cord development. Exp Neurol 274, 90-99.
Wilson, L., Maden, M., 2005. The mechanisms of dorsoventral patterning in the vertebrate neural tube. Dev Biol 282, 1-13.
Windrem, M.S., Schanz, S.J., Morrow, C., Munir, J., Chandler-Militello, D., Wang, S., Goldman, S.A., 2014. A competitive advantage by neonatally engrafted human glial progenitors yields mice whose brains are chimeric for human glia. J Neurosci 34, 16153-16161.
Wittler, L., Kessel, M., 2004. The acquisition of neural fate in the chick. Mech Dev 121, 1031-1042.
Wujek, J.R., Lasek, R.J., 1983. Correlation of axonal regeneration and slow component B in two branches of a single axon. J Neurosci 3, 243-251.
Xia, H., Sun, X., Liu, D., Zhou, Y., Zhong, D., 2016. Oriented growth of rat Schwann cells on aligned electrospun poly(methyl methacrylate) nanofibers. J Neurol Sci 369, 88-95.
Xiong, L.L., Liu, F., Deng, S.K., Liu, J., Dan, Q.Q., Zhang, P., Zou, Y., Xia, Q.J., Wang, T.H., 2017. Transplantation of Hematopoietic Stem Cells Promotes Functional Improvement Associated with NT-3-MEK-1 Activation in Spinal Cord-Transected Rats. Front Cell Neurosci 11, 213.
Yamada, T., Pfaff, S.L., Edlund, T., Jessell, T.M., 1993. Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell 73, 673-686.
Yamada, T., Placzek, M., Tanaka, H., Dodd, J., Jessell, T.M., 1991. Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. Cell 64, 635-647.
Yan, J., Welsh, A.M., Bora, S.H., Snyder, E.Y., Koliatsos, V.E., 2004. Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. J Comp Neurol 480, 101-114.

Yan, X., Liu, J., Luo, Z., Ding, Q., Mao, X., Yan, M., Yang, S., Hu, X., Huang, J., Luo, Z., 2010. Proteomic profiling of proteins in rat spinal cord induced by contusion injury. Neurochem Int 56, 971-983.

Yang, Z., Bramlett, H.M., Moghieb, A., Yu, D., Wang, P., Lin, F., Bauer, C., Selig, T.M., Jaalouk, E., Weissman, A.S., Rathore, D.S., Romo, P., Zhang, Z., Hayes, R.L., Wang, M.Y., Dalton Dietrich, W., Wang, K.K., 2017. Temporal Profile and Severity Correlation of a Panel of Rat Spinal Cord Injury Protein Biomarkers. Mol Neurobiol.

Yntema, C., Hammond, W., 1954. The Origin of Intrinsic Ganglia of Trunk Viscera from Vagal Neural Crest in the Chick Embryo. Journal of Comparative Neurology 101, 515-541.

Yoshihara, M., Hayashizaki, Y., Murakawa, Y., 2017. Genomic Instability of iPSCs: Challenges Towards Their Clinical Applications. Stem Cell Rev 13, 7-16.

Young, H.M., Bergner, A.J., Muller, T., 2003. Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. J Comp Neurol 456, 1-11.

Young, H.M., Bergner, A.J., Simpson, M.J., McKeown, S.J., Hao, M.M., Anderson, C.R., Enomoto, H., 2014. Colonizing while migrating: how do individual enteric neural crest cells behave? BMC Biol 12, 23.

Young, H.M., Jones, B.R., McKeown, S.J., 2002. The projections of early enteric neurons are influenced by the direction of neural crest cell migration. J Neurosci 22, 6005-6018.

Young, H.M., Turner, K.N., Bergner, A.J., 2005. The location and phenotype of proliferating neural-crest-derived cells in the developing mouse gut. Cell Tissue Res 320, 1-9.

Yu, P., Wang, H., Katagiri, Y., Geller, H.M., 2012. An in vitro model of reactive astrogliosis and its effect on neuronal growth. Methods Mol Biol 814, 327-340.

Yuan, X.B., Jin, Y., Haas, C., Yao, L., Hayakawa, K., Wang, Y., Wang, C., Fischer, I., 2016a. Guiding migration of transplanted glial progenitor cells in the injured spinal cord. Sci Rep 6, 22576.

Yuan, Y., Yang, Y., Tian, Y., Park, J., Dai, A., Roberts, R.M., Liu, Y., Han, X., 2016b. Efficient long-term cryopreservation of pluripotent stem cells at -80 degrees C. Sci Rep 6, 34476.

Yuan, Y.M., He, C., 2013. The glial scar in spinal cord injury and repair. Neurosci Bull 29, 421-435.

Yue, J.K., Chan, A.K., Winkler, E.A., Upadhyayula, P.S., Readdy, W.J., Dhall, S.S., 2016. A review and update on the guidelines for the acute management of cervical spinal cord injury - Part II. J Neurosurg Sci 60, 367-384.

Zhang, N., Yin, Y., Xu, S.J., Wu, Y.P., Chen, W.S., 2012. Inflammation & apoptosis in spinal cord injury. Indian J Med Res 135, 287-296.

Zhang, X., Bhattacharyya, S., Kusumo, H., Goodlett, C.R., Tobacman, J.K., Guizzetti, M., 2014. Arylsulfatase B modulates neurite outgrowth via astrocyte chondroitin-4-sulfate: dysregulation by ethanol. Glia 62, 259-271.

Zhao, J., Sun, W., Cho, H.M., Ouyang, H., Li, W., Lin, Y., Do, J., Zhang, L., Ding, S., Liu, Y., Lu, P., Zhang, K., 2013. Integration and long distance axonal regeneration in the central nervous system from transplanted primitive neural stem cells. J Biol Chem 288, 164-168.

Zhao, L.R., Duan, W.M., Reyes, M., Keene, C.D., Verfaillie, C.M., Low, W.C., 2002. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174, 11-20.

Zhao, R.R., Fawcett, J.W., 2013. Combination treatment with chondroitinase ABC in spinal cord injury--breaking the barrier. Neurosci Bull 29, 477-483.
Zhao, Y., Zuo, Y., Wang, X.L., Huo, H.J., Jiang, J.M., Yan, H.B., Xiao, Y.L., 2015. Effect of neural stem cell transplantation combined with erythropoietin injection on axon regeneration in adult rats with transected spinal cord injury. Genet Mol Res 14, 17799-17808.
Zhou, X.H., Ning, G.Z., Feng, S.Q., Kong, X.H., Chen, J.T., Zheng, Y.F., Ban, D.X., Liu, T., Li, H., Wang, P., 2012. Transplantation of autologous activated Schwann cells in the treatment of spinal cord injury: six cases, more than five years of follow-up. Cell Transplant 21 Suppl 1, S39-47.
Zhou, Y.J., Liu, J.M., Wei, S.M., Zhang, Y.H., Qu, Z.H., Chen, S.B., 2015. Propofol promotes spinal cord injury repair by bone marrow mesenchymal stem cell transplantation. Neural Regen Res 10, 1305-1311.
Zhuo, M., Gebhart, G.F., 1991. Tonic cholinergic inhibition of spinal mechanical transmission. Pain 46, 211-222.
Ziegler, M.D., Hsu, D., Takeoka, A., Zhong, H., Ramon-Cueto, A., Phelps, P.E., Roy, R.R., Edgerton, V.R., 2011. Further evidence of olfactory ensheathing glia facilitating axonal regeneration after a complete spinal cord transection. Exp Neurol 229, 109-119.